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GENIC INDUCTION OF AN INHERITED CYTOPLASMIC DIFFERENCE

By M. M. Rhoades

Department of Botany, Columbia University

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Although many biologists consider the genes to be the sole determiners of heredity, there are those who feel that the cytoplasm contains a system of independent entities which in some cases controls the expression of certain characteristics. The terms genome and plasmone have been used to denote the system of genes and of cytoplasmic entities, respectively. Although the development of chlorophyll has been shown in hundreds of cases to be under genic control, there are a number of instances where chlorophyll variegation is inherited independently of the genome. These chlorophyll variegations, transmitted through the female line only, constitute the most compelling evidence for cytoplasmic inheritance. In these cases the physical entities in the cytoplasm are known to be the plastids; in other cases of plasmatic inheritance the nature of the entities in the cytoplasm can only be conjectured.

In maize there are more than one hundred cases where the development of chlorophyll is under genic control. Two examples of cytoplasmically inherited chlorophyll variegation have been reported,[1, 2] as has one case of the cytoplasmic inheritance of male sterility.[3] Among the chlorophyll characters in maize which are genically controlled is that of iojap. Maize plants homozygous for the recessive gene iojap (*ij*) exhibit a chlorophyll striping or variegation.[4] Considerable variation is found in the extent and pattern of the green and white areas of the leaves and culm. The *ij* gene is situated in chromosome 7; its position in the linkage group has been determined with some precision through 3-point linkage tests.[5]

When *ij ij* plants are used as the pollen parent in crosses with normal (*Ij Ij*) individuals, the F_1 progenies consist wholly of green plants—i.e., the *ij* allele is completely recessive to the normal *Ij* allee. However, as Jenkins first noted, when *ij ij* individuals are used as the female parent

in crosses with *Ij Ij* plants, the F_1 progenies often contain white and/or striped seedlings in addition to the expected green ones. Great variations are found in the proportions of white and striped seedlings; sometimes all the seedlings from an F_1 ear are white. (The *ij* plants used in these experiments arose in the F_2 generation of the cross *Ij* ♀ × *ij* ♂.)

A cytological examination of the white regions of *ij ij* plants disclosed that the plastids of the mesophyll cells not only lacked chlorophyll but also were much smaller than were the plastids of the normal green areas. It appears, therefore, that the *ij* gene is able to induce a modification in the plastid. That these modified plastids have a genetic continuity, which is not affected by the genic constitution and therefore may be considered as mutations is suggested by the observations that the F_1 plants of *Ij ij* constitution may be white or striped. If a white F_1 seedling arises, it is assumed that the cytoplasm of the egg cell carried only plastid primordia of the mutated type, while striped individuals are assumed to have come from an egg cell whose cytoplasm contained both normal and mutated plastic primordia, with somatic segregation of the two plastic types giving the striped pattern. Support for the above hypothesis was obtained from a cytological examination of the white areas of *Ij ij* plants which showed them to have a type of plastid similar to that found in the white areas of the parent *ij ij* plant. Furthermore, both types of plastids were found in certain green cells, adjacent to the white regions.

The correctness of the hypothesis that in *ij ij* plants an irreversible mutation is induced in the plastid by the *ij* allele is proved by the following evidence. Striped F_1 plants from the cross *ij ij* ♀ × *Ij Ij* ♂ were crossed with pollen from unrelated *Ij Ij* individuals. The backcross progenies were obtained from such crosses. Genetically each population consisted of equal numbers of *Ij Ij* and *Ij ij* plants. Some of the back-crossed ears on F_1 striped plants gave progenies consisting entirely of green seedlings. Such ears, it is argued, arose from green sectors of the F_1 plants and hence the egg cytoplasm carried normal plastid primorida. Other ears gave rise to progenies with varying percentages of green, striped and white seedlings. Here it is assumed that the ears came from both green and white tissue and therefore some egg cells had normal plastids while some had mutated plastid primordia. Occasionally all the progeny from a back-crossed ear consisted of white seedlings only and consequently came from a white sector of the parent plant. These all white populations are the most instructive. Half of the individuals in such populations are homozygous for the *Ij* allele and half are heterozygous. It is a pertinent fact that in *Ij Ij* cells the mutant plastid continues to give rise to mutant plastids; there is no control by nuclear factors on the type of plastid. Although induced by a nuclear factor,

the *ij* gene, the mutated plastid, like a Frankenstein monster, is no longer under the control of its maker.

The data given here are of direct interest in connection with the nucleus-plasma problem, but they may have some significance in the field of both normal and abnormal growth and differentiation. The basis of cellular differentiation is one of the great problems of biology. All nuclei of an organism presumably have the same genic constitution, and yet morphological and physiological differences arise. That the maintenance of these differences is not entirely determined by the differing local conditions is shown by the persistence of certain specificities when cell multiplication of isolated differentiated cells occurs in tissue culture. The view that cellular differentiation is cytoplasmic seems to require that the cytoplasm contains elements of a hypothetical nature which are modified by interaction with nuclear products. In the case reported here a known constituent of the cytoplasm, the plastid, has been modified by a nuclear factor and is transmitted thereafter by cytoplasmic heredity.

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GENE AND CYTOPLASM. I. THE DETERMINATION AND INHERITANCE OF THE KILLER CHARACTER IN VARIETY 4 OF PARAMECIUM AURELIA[1]

BY T. M. SONNEBORN[2]

DEPARTMENT OF ZOÖLOGY, INDIANA UNIVERSITY

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The present paper reports a previously unknown system of relations between a gene and a cytoplasmic substance, both of which are required for the development of an hereditary character. When some of the cytoplasmic substance is present, the gene controls its continued production; but when the cytoplasmic substance is absent, the gene cannot initiate its production. Addition of the cytoplasmic substance to an organism, lacking the character dependent on it, but containing the required gene, results in the continued production of the substance, in the development of the character determined by the combined presence of gene and cytoplasmic substance, and in the hereditary maintenance of the character in successive generations.

The pair of characters whose determination and inheritance are to be analyzed are designated "killer" and "sensitive." They are found in diverse races of variety 4 of *Paramecium aurelia*. Of the four available races of this variety, only race 51 is a killer; the other three races (29, 32 and 47) are sensitive. Fluid in which the killer race has lived kills individuals of the sensitive races. The killing of sensitive animals is preceded by characteristic morphological aberrations, particularly by shifting of the posterior part of the body to the aboral side. The killer and sensitive characters are alternatives in inheritance and never exist together in the same individual. In practice, the character of an unknown clone is tested by mixing samples of it (1) with the killer race 51, and (2) with a sensitive race. If the characteristic abnormalities and corpses are produced in mixture (1), the unknown is sensitive; if produced in mixture (2), it is a killer. Sensitive animals begin to show abnormalities only after at least four hours of subjection to the killer fluid. It is possible to cross killers with sensitives if they begin to mate soon after they are brought together, and this is readily accomplished by bringing together opposite mating types when in sexually reactive condition. If the conjugant pairs are removed to fresh culture fluid soon after they unite and the two members of each pair are put into separate culture vessels soon after conjugation has been completed, the sensitive member of the pair is never injured by the contact with a killer during mating.

In the original races, the characters killer and sensitive are invariably inherited. All the vegetative and sexual progeny within race 51 are killers. All the vegetative and sexual progeny within the sensitive races are sensitive. Only the sensitive race 32 is reported upon in this paper; results with other sensitive races bring out certain further important points which will be set forth in the next paper of this series.

When the pure killer race 51 is crossed to the pure sensitive race 32, the two exconjugants of each pair produce phenotypically different clones: one is a killer and the other is sensitive. By marking the parents, it is readily demonstrated that the F_1 killer clones are those that derive their cytoplasm from the killer parent and the F_1 sensitive clones are those with cytoplasm from the sensitive parent. This result is totally unexpected and requires explanation because, as is well known, the nuclear processes during conjugation are such that the two mates, after reciprocal fertilization, have the same genotype and should produce clones alike in their hereditary characters. The following experiments were designed to provide the required explanation.

Experiment 1: Autogamy in F_1 Killers.—Ultimate genic control of the alternative characters is indicated by observations on the results of autogamy in one of the two classes of F_1 clones, the killers. Autog-

amy[3, 4, 5] is a process in which identical, haploid, gamete nuclei in a single unmated cell unite to produce a diploid syncaryon from which all subsequent nuclei of the clone are derived. Hence all clones derived from autogamous individuals are necessarily homozygous and, when heterozygotes undergo autogamy, half become homozygous for one allele and half for the other. Of 306 F_1 killers that went through autogamy, 167 produced clones of killers and 139 produced clones of sensitives. The segregation ratio is reasonably close to the theoretical 1:1 ratio (deviation 14, standard error 8.7) and so indicates that the F_1 killers were heterozygous for a pair of alleles determining the alternative characters.

Experiments 2, 3, 4 and 5: Further Fertilizations within Each of the Two Classes Obtained in Experiment 1.—If the F_1 killers were heterozygotes, the two classes that segregated from them at autogamy (expt. 1) should be the two possible classes of homozygotes. Further breeding tests confirmed this: the sensitive F_2 clones yielded only sensitive progeny after further autogamies (expt. 2) and after conjugation with each other (expt. 3); the killer F_2 clones yielded only killer progeny after further autogamies (expt. 4) and after conjugation with each other (expt. 5). Hence the two classes of F_2 clones obtained in approximately equal numbers at autogamy in F_1 killers (expt. 1) are pure breeding or homozygous for the alternative characters. The genic determination of these characters was further tested in experiment 6.

Experiment 6: Crosses between Different Clones of F_1 Killers.—If the F_1 killers are heterozygotes, conjugation between two such clones should yield the usual F_2 ratio of 3:1 and should show which allele is dominant. From 443 pairs of conjugants the 652 clones from the two members of 326 pairs were killers and the 234 clones from the two members of 117 pairs were sensitives. This agreement with the theoretical 3:1 ratio (deviation $6^1/_4$, standard error 9) confirms the heterozygosity of the F_1 killers and shows that the killer gene (K) is dominant over its sensitive allele (k).

Since F_1 killers are heterozygous (K/k), their pure breeding parent races must have the two possible homozygous combinations: the killer race is K/K and the sensitive race is k/k. As the killer gene is dominant (expt. 6), *all* the F_1 should be heterozygous killers; but this condition has been demonstrated only for one of the two F_1 clones from each pair of hybrid exconjugants. The other is not a killer, but sensitive (see p. 330). Is the disagreement here merely phenotypic or genotypic also? Experiments 7 and 8 answer this question.

Experiment 7: Cross of F_1 Sensitives to F_1 Killers.—In order to discover the genotype of the F_1 sensitives, they were crossed to F_1 killers known (expts. 1–6) to be heterozygotes. From this cross were obtained 294 conjugant pairs yielding a killer clone from one member and a sensitive

clone from the other member of each pair: and 126 conjugant pairs yielding sensitive clones from both members of each pair. The meaning of this result is best brought out by considering first those clones that derived their cytoplasm from the F_1 killers, i.e., one member of each pair of exconjugant clones.

These cytoplasmic descendants of the F_1 killers included 294 killer clones and 126 sensitive clones. This is closer to a theoretical 3:1 ratio (deviation 21, standard error 8.9) than to any other simple genetic ratio. In the detailed paper to be published later it will be shown that the small discrepancy not due to sampling error is due to the occurrence, in a small percentage of the united pairs, of a process differing from normal conjugation. This process is essentially double autogamy or, as it has been called,[6] cytogamy. In most crosses it can readily be detected and the data can be corrected for it; but, in crosses of the type under discussion, its detection is so laborious as to be quite impracticable.

The 3:1 ratio in this group shows that both parents were heterozygotes, as in crosses between F_1 killers (expt. 6). One parent was a clone of F_1 killers (known to be heterozygotic) and the other parent was a clone of F_1 sensitives. Hence, the F_1 sensitives must also have been heterozygotic (K/k) and this agrees with expectations from the breeding experiments 1 to 6. The disagreement with expectation is merely in their sensitive phenotype. The killer gene K, dominant in race 51 cytoplasm (expt. 6), is certainly not dominant in race 32 cytoplasm.

Further information on the behavior of gene K in race 32 cytoplasm is provided by consideration of the phenotypes of the other member of each pair of exconjugant clones produced in experiment 7, namely, those deriving their cytoplasm from the F_1 sensitives. *All* of the 420 clones of this group were sensitive. From the now known genotypes of their parents, $K/k \times K/k$, one-fourth of these 420 clones should be k/k and sensitive, one-half should be K/k and sensitive (because, as shown above, K is not dominant in race 32 cytoplasm) and one-fourth should be K/K. The fact that no killer clones were obtained in this group indicates that even the K/K clones were sensitive. In other words, the killer gene K is completely unable to produce the killer phenotype in race 32 cytoplasm. Experiments 8, 9 and 10 confirm this conclusion.

Experiment 8: Autogamy in F_1 Sensitive Clones.—As in the case of autogamy in heterozygous F_1 killer clones (expt. 1), autogamy in heterozygotic F_1 sensitive clones should yield K/K and k/k clones in a ratio of 1:1. But as the F_1 sensitive clones and their autogamous progeny derive their cytoplasm from race 32 and as the killer gene K is unable to produce the killer phenotype in this cytoplasm (expt. 7), both classes of exautogamous clones should be sensitive. In agreement with this, all of the 148 exautogamous clones from F_1 sensitives were sensitive.

Experiment 9: Crosses of F_2 Sensitive Clones (Obtained by Autogamy from F_1 Sensitives) to F_1 Killers.—It is practically impossible to test whether the 1:1 ratio predicted in experiment 8 is actually obtained because each clone would have to be separately tested for genotype by elaborate breeding experiments. More important than the ratio is the question of whether there are in fact produced, as predicted, sensitive clones homozygous for the killer gene. Therefore, only a few of the exautogamous sensitive clones from experiment 8 were fully tested to see if any had the predicted K/K genotype. The test consisted in crossing them to heterozygous F_1 killers. In this cross, any tested clone that contains the recessive gene k either in homozygous or heterozygous condition would yield some pairs in which both members produce sensitive clones; but if the tested clone is homozygous for the killer gene K, all the pairs of conjugants obtained in the cross to F_1 killers would yield a killer clone from one member of the pair and a sensitive clone from the other. Among the few clones tested, three gave the latter result. For example, 36 pairs were obtained from the cross of one clone to F_1 killers and from each pair there arose one clone of killers and one clone of sensitives. The other two were tested on an even larger scale and gave the same result. Hence, some of the exautogamous clones obtained from F_1 sensitive parents are homozygous for the killer gene though phenotypically sensitive. This confirms the conclusion drawn from experiment 7 that the killer gene is unable to determine the killer phenotype in race 32 cytoplasm even when it is present in homozygous condition.

Experiment 10: Sensitive Clones Homozygous for the Killer Gene Retested after the Passage of Several Months.—In order to exclude the possibility that the sensitivity of the K/K clones with race 32 cytoplasm was due either to the delayed action of the K gene or to the mutation of K to k in race 32 cytoplasm, these clones were retested at intervals of 2, 4 and 6 months. The tests showed that the sensitive phenotype and the K/K genotype were maintained. (The tests were the same as the one employed in experiment 9.) During the six months that these clones were cultured, many successive sexual generations must have occurred, for autogamies recur at intervals of 3 to 7 days in mass cultures of variety 4. Hence the gene K remains constant and is not only temporarily but permanently incapable of determining the killer phenotype in race 32 cytoplasm.

These results raise the question of cytoplasmic inheritance. In cytoplasm of race 51, the gene K determines the killer character; in cytoplasm of race 32, the same gene does not determine the killer character. This difference in the effect of gene K in different cytoplasms persists through many sexual generations, presumably without limit. Does this warrant the conclusion that the cytoplasms of the two races possess hereditary

differences that are independent of the genes? Experiment 11 answers this question.

Experiment 11: Cross of F_2 Sensitive Clones (Derived by Autogamy from F_1 Killers) to the Killer Race 51.—Experiments 1, 4 and 5 showed that half of the clones obtained by autogamy from F_1 killers were sensitive because they were homozygous for the sensitive gene k. The cytoplasm of these clones is derived from race 51. If the property of race 51 cytoplasm which permits the killer gene K to determine the killer character is inherited independently of the genes, reintroduction of gene K into these sensitive clones should result in the restoration of the killer character. The gene K was put back into them by mating them to the homozygous killer race 51; but the killer character failed to develop. In all of the 96 pairs of conjugants, the clone produced by the exconjugant deriving its cytoplasm *directly* from the killer parent remained a killer; but the clone produced by the exconjugant deriving its cytoplasm *indirectly* from race 51, through a sensitive F_2 clone, remained sensitive. Hence, when the gene K is replaced by its sensitive allele, k, the cytoplasm of race 51 becomes, like the cytoplasm of race 32, incapable of developing the killer phenotype when gene K is reintroduced into it. The property of race 51 cytoplasm which permits the killer gene to determine the killer character is thus not inherited independently of the genes, but is dependent on the uninterrupted presence of the gene K.

This experiment shows that the killer character depends on the combined presence of the killer gene K and something else. The other factor, when present, is reproduced under the influence of gene K and ceases to be reproduced when gene K is absent. Moreover, the failure of the killer character to develop in sensitive clones into which gene K has been introduced shows that gene K is unable to initiate the production of this other essential factor and that the latter is not carried over from one mate to the other during conjugation. In other words, it is not present in the "male," migratory, gamete nucleus at the time of fertilization. Experiments 12 and 13 were designed to throw light on the location within the cell of the essential factor other than gene K.

Experiment 12: Transfer of the Other Factor from one Cell to Another.—Under certain conditions, not yet entirely known,[7] pairs of conjugants in variety 4 either remain united unduly long or permanently. At the normal time for separation of the mates, they separate everywhere except in the region of the paroral cones where a thin connecting band of cytoplasm appears. After prolonged union in this way, in some pairs the mates separate completely; but in others the band of union increases in width and the mates remain permanently united, though normal single animals are given off from the separated regions at the first few fissions. Such connections between mates provide opportunity for the transfer

of material from one to the other, especially in those pairs that develop a broad connecting band. In crosses between killers and sensitive clones with the killer gene *K*, when a cytoplasmic connection was established between the mates, the normal single animals produced from *both* of them yielded killer clones. Hence the formerly sensitive mate must have produced a clone with the killer phenotype and this result is correlated with the possession of the gene *K* plus temporary cytoplasmic continuity with a killer animal. In the same crosses, when separation of the mates occurred at the normal time, each mate remained phenotypically unchanged: one produced a clone of killers and the other a clone of sensitives. Hence the transformation of the sensitive into a killer clone in the cytoplasmically united pairs must have been due to the transfer from the killer to the sensitive mate of the material required in addition to gene *K* for the development of the killer phenotype. Moreover, of the gamete nuclei, syncaryon and derivatives of the syncaryon, only the migratory gamete nucleus goes across from one mate to the other in the cytoplasmically united pairs, and previous experiments have shown that the migratory nucleus does not carry the essential material with it; therefore this material at this time must be outside of these nuclei. The material could be in either the cytoplasm or the many pieces into which the old, disintegrating macronucleus has broken down, for these are both free to migrate from mate to mate across the broad cytoplasmic connecting band. There is, however, no known direct connection between the pieces of the old macronucleus and the new nuclei formed from the products of the syncaryon; and the old macronuclear pieces soon disappear, while the killer character is permanent and hereditary. Hence the essential material must be at least for a time outside the nuclei in the cytoplasm. There is as yet no evidence concerning the question as to whether it is ever in the nuclei. The substance whose continued production is controlled by gene *K* and whose presence is required for the development of the killer phenotype may therefore be designated as the killer cytoplasmic factor or substance. Whether this is the same as the substance that produces the killing action on sensitive cells or a precursor of it remains to be discovered.

Experiment 13: Demonstration of the Killer Cytoplasmic Factor in Cells That Have Just Lost the Killer Gene.—The killer cytoplasmic factor was shown in experiment 11 to disappear from cells that lose the killer gene *K*. However, only if this factor did not exist in the cytoplasm but was always indissolubly connected with gene *K* would its disappearance be expected to coincide exactly with the loss of gene *K;* otherwise, its disappearance should follow loss of gene *K* by an appreciable amount of time. If it could be detected in the cell for a considerable period after gene *K*

is replaced by k, this would provide further evidence for its cytoplasmic localization.

The method of detection employed was essentially the same as the one used in experiment 11: F_2 sensitives (k/k) that had arisen from F_1 killers (K/k) at autogamy were mated to the killer race 51 (K/K). In experiment 11 this cross was made several days after the genotype had changed at autogamy from K/k to k/k. At that time there was no evidence of the presence of the cytoplasmic factor, for return to the K/k genotype did not result in the development of the killer phenotype. In the present experiment, the same cross was made at intervals of only two to five fissions (one to two days) after the F_1 killer (K/k) had changed to an F_2 sensitive (k/k) at autogamy. Description of the laborious technique involved in bringing about conjugation so early in the history of a clone of known constitution will be reserved for the full paper to appear later.

Altogether 21 such F_2 sensitive clones were induced to conjugate with the killer race 51 within five fissions (2 days) after their origin at autogamy. Three of the four that conjugated two or three fissions after autogamy, four of the ten that conjugated three or four fissions after autogamy, and one of the seven that conjugated five fissions after autogamy, making a total of eight crosses, yielded clones of killers from *both* members of a pair of conjugants. Hence the sensitive parent, as well as the killer parent, must have yielded a clone of killers in these eight cases; and the cytoplasmic factor must still have been present. The data also indicate that the cytoplasmic factor is present in fewer and fewer cells with increase of time and number of fissions since loss of gene K, until, as shown in experiment 11, it has completely disappeared after a few days. The period during which the cytoplasmic factor remains after the gene for its production is removed corresponds closely to the period previously found[8,9] for the "cytoplasmic lag" in change of phenotype following other changes in hereditary constitution in *P. aurelia*. Comparable gradual loss of a gene-controlled cytoplasmic factor may be involved in many or all such situations.

Both experiments 12 and 13 show: (1) that the factor required in addition to gene K for development of the killer phenotype is present in the cytoplasm of killer cells; (2) that gene K determines the continued production of this cytoplasmic factor, for sensitive cells converted into killers became permanently and hereditarily so; and (3) that this can be accomplished when the cytoplasmic factor is at least initially outside the nucleus in the cytoplasm. Both experiments also show (4) that there was present in one cell at least twice as much of the cytoplasmic factor as required by gene K to enable it to produce more. This was shown in experiment 12 by the fact that the killer member of the cyto-

plasmically united pairs provided enough of the cytoplasmic factor to enable genes in both cells of the pair to produce more; and in experiment 13 by the fact that in each of three different crosses of exautogamous clones to race 51, there were obtained *two* pairs of conjugants yielding killer clones from both members of the pair. Hence, in each of these three clones there was present after gene K was lost enough of the cytoplasmic factor so that it could be distributed to two daughter cells and still have in each enough to bring about the production of more cytoplasmic factor when gene K entered the cell.

The preceding 13 experiments provide the basic information required for discovery of the system of determination and inheritance of the killer and sensitive characters. Many other breeding experiments have been performed to test further the validity of the conclusions drawn in the preceding pages. Space does not permit a detailed account, but the crosses and observed results in some of the more important experiments are listed below.

Experiment 14.—Cross of F_1 killers (K/k) to race 32 (k/k); 210 pairs of conjugants. Result: 116 pairs yielded sensitive clones from both members of the pair; 94 pairs yielded a killer clone from one member and a sensitive clone from the other.

Experiment 15.—Cross of F_1 killers (K/k) to race 51 (K/K); 104 pairs of conjugants. Result: killer clone from each member of every pair.

Experiment 16.—Cross of F_1 sensitive (K/k) by F_1 sensitive (K/k); 431 pairs of conjugants. Result: all 862 clones sensitive.

Experiment 17.—Cross of F_1 sensitive (K/k) to race 32 (k/k); 200 pairs of conjugants. Result: all 400 clones sensitive.

Experiment 18.—Cross of F_1 sensitive (K/k) to race 51 (K/K); 107 pairs of conjugants. Result: in every pair, one member produced a sensitive clone and the other produced a killer clone.

In every experiment the observed results are those required by the conclusions drawn from the first 13 experiments.

The determination and inheritance of the alternative characters killer and sensitive in races 51 and 32 appear therefore to involve the following system. Killer depends upon the combined presence of the dominant gene K and a cytoplasmic substance. The continued production of this substance depends upon gene K; but gene K is unable to initiate its production when none is present. The alternative character sensitive invariably develops regardless of genic constitution when the cytoplasmic substance is absent. A recessive allele of K is unable to determine the continued production of the cytoplasmic substance even when some of it is present. All of the nine pairs of characters examined in five varieties of *P. aurelia* show the same peculiar division of the F_1 into two classes. This suggests that a comparable system of determination and inheritance

is widespread in this species. The significance of the system in relation to a number of problems of biology will be discussed in the next paper of this series.

[1] Contribution No. 324 from the Department of Zoölogy, Indiana University.

[2] Aided by a grant from the Rockefeller Foundation. The author is indebted to Ruth V. Dippell for invaluable assistance in conducting the experiments.

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GENE AND CYTOPLASM. II. THE BEARING OF THE DETERMINATION AND INHERITANCE OF CHARACTERS IN PARAMECIUM AURELIA ON THE PROBLEMS OF CYTOPLASMIC INHERITANCE, PNEUMOCOCCUS TRANSFORMATIONS, MUTATIONS AND DEVELOPMENT[1]

By T. M. Sonneborn[2]

Department of Zoölogy, Indiana University

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The first paper[3] of this series demonstrated that the character "killer" in variety 4 of *Paramecium aurelia* is dependent upon a cytoplasmic substance which normally fails to accompany the "male" gamete nucleus as it passes from one mate to the other during conjugation. The continued production of this determining cytoplasmic substance depends, however, on a dominant gene, *K;* replacement of *K* by its recessive allele, *k*, results in the disappearance of the active cytoplasmic substance. Nevertheless, the gene *K* is unable to initiate production of the cytoplasmic substance; introduction of *K* into a sensitive cell is not followed by development of the killer character. But if a non-killer (sensitive) cell containing gene *K* is supplied with some cytoplasm from a killer cell, or if a genotypically sensitive cell containing the cytoplasmic factor is supplied with gene *K*, the gene *K* controls the continued production of the killer cytoplasmic substance. This system of determination and inheritance appears to be typical for all characters in most varieties of *P. aurelia*. The preceding facts may have important applications to other fields of biology. The present paper attempts to point out some of these.

1. Cytoplasmic Inheritance.—Inheritance through the cytoplasm (aside from plastid inheritance) has been reported by a number of investigators

of mosses and flowering plants; this work has been critically reviewed by Correns[4] and others. The observations on which the interpretation of cytoplasmic inheritance is based are closely parallel to some of those made on Paramecium. Full analysis shows that the interpretation in terms of cytoplasmic inheritance in Paramecium is an illusion and untenable. This raises the question as to whether the same interpretation based on similar evidence in plants is sound.

In plants the fundamental observations are: (1) reciprocal crosses yield different results; (2) the differences persist through subsequent generations in the female line of descent, even when all or nearly all of the genes of the female parent have been replaced by genes from the male parent.

Observations on *P. aurelia* precisely like the first one mentioned in the preceding paragraph were reported in the first paper[3] of this series. Each pair of conjugants between diverse clones or races constitutes a pair of reciprocal crosses: One member of the pair of conjugants contains all the cytoplasm and one gamete nucleus from one parent and only a gamete nucleus from the other parent; the other member of the pair of conjugants has its cytoplasm and one gamete nucleus from the other parent and only a gamete nucleus from the first parent. These reciprocal crosses yield different results when the killer race is crossed to a sensitive race: the one in which the cytoplasm comes from the killer race produces a clone of killers and the one in which the cytoplasm comes from the sensitive race produces a clone of sensitives. Reciprocal crosses in Paramecium, as in plants, yield different results.

The parallel between the plants and Paramecium with respect to the second fundamental observation in the work on cytoplasmic inheritance is best shown by certain additional results not set forth in the first paper of this series. If, instead of using the sensitive race 32, reported upon in the previous paper, the sensitive race 47 is employed in crosses to the same killer race 51, the same results are obtained in the F_1 generation: reciprocal crosses give different results; those with cytoplasm from the killer race are killers and those with cytoplasm from the sensitive race are sensitive. But very different results follow in subsequent generations. The F_1 killers, though they have genes from both races, show no segregation of these traits in any breeding tests. In both autogamy and in backcrosses the progeny that derive their cytoplasm from the killer race remain killers. Likewise, the progeny of the F_1 sensitive clones that derive their cytoplasm from the sensitive race remain sensitive.

The preceding breeding experiments show, as do the comparable experiments with plants, that the hereditary differences under examination are due to cytoplasmic and not to genic differences. This was further demonstrated[3] directly by providing an opportunity for transfer of cyto-

plasm from a killer to sensitive cell through a cytoplasmic connecting bridge between them. When cytoplasm of the killer race 51 gets in this way into sensitive animals of an appropriate genotype, the latter are transformed from the sensitive to the killer condition and the new character is inherited in all subsequent generations by those progeny that obtain their cytoplasm from the transformed individuals. This evidence makes the demonstration of the determinative influence of the cytoplasm complete. The persistence of the effect through the passage of generations would appear to justify the conclusion of cytoplasmic inheritance.

This conclusion would have been unavoidable if only races 47 and 51 had been available. Only the extremely good fortune of having the race 32 for use in further analysis prevented me from falling into this error. As set forth in the preceding paper,[3] race 32 differs from race 51 not only in its cytoplasm (as does also race 47), but also in a gene which controls the cytoplasmic difference. When the killer gene *K* in race 51 is replaced by the sensitive allele *k* from race 32, the cytoplasmic factor determining the killer condition ceases to be reproduced. The phenomenon under analysis is thus not cytoplasmic inheritance, but continued production of a cytoplasmic substance under the influence of the single gene *K*. The false conclusion drawn from analyses limited to the sensitive race 47 is a consequence of the fact that this sensitive race, like the killer race 51, possesses the killer gene *K*. As previously shown,[3] this gene cannot initiate production of the killer cytoplasmic substance, but merely controls its continued production when some is already present. The full proof, from a variety of experiments, that the race 47 contains the killer gene *K* will be given in a later paper. For the present, the facts already given above (absence of segregation in further breeding of F_1 killers) will suffice.

The preceding analysis shows that results such as those obtained with the plant material and with Paramecium (in the crosses not involving race 32) justify the conclusion that the differences observed in reciprocal crosses and subsequent generations are due to cytoplasmic and not to genic differences; they do not, however, justify the conclusion that cytoplasmic inheritance, in the sense of independent, self-multiplying, cytoplasmic determinants, is involved. The cytoplasmic differences may be perpetuated, not by independent cytoplasmic determinants, but by genes which are alike in the two forms crossed; and these genes, like the killer gene, may be unable to initiate production of the cytoplasmic substances involved. Only by means of specially favorable material can the genic control of the cytoplasmic substances be discovered.

2. Environmental Control of Genetic Characters in Bacteria.—The phenomena of the inheritance of the antigenic properties of Pneumococcus constitute one of the most fully known examples of environmental control of hereditary characters. The main facts have recently been reviewed

by Heidelberger.[5] More than 50 distinct antigenic types of Pneumococcus are known. The character of each of these types is normally inherited within a clone; but by appropriate procedures a number of types have been converted into others and probably all conceivable transformations of one into another are possible. The conversion is accomplished by reducing one type into a non-specific form in which the capsule with its specific antigens (polysaccharides) are lacking, and then growing the non-specific form in the presence of heat-killed cells of the type to which conversion is desired. Thereupon, the form lacking a capsule acquires one which contains the type of polysaccharide antigen characteristic of the heat-killed cells. The essential material required for successful conversion is not the polysaccharide itself, but some other component of the heat-killed cell containing it.

The Pneumococcus situation may be compared with the killer situation in Paramecium. In the latter, transfer of a cytoplasmic substance from a killer cell to a sensitive cell containing gene *K* will result in the continued production of this substance and the killer phenotype which depends on it. Possibly all or most strains of Pneumococcus contain the gene or genes (or gene-like materials) required to control the continued production of each of the 50 different polysaccharides, and, as in Paramecium, when an appropriate substance essential for the synthesis of the polysaccharide is added to a cell that lacked it, the "gene" will determine its continued production. As in Paramecium, the gene seems to be unable to initiate its production, but can continue it when the proper substance is provided to start the gene going. The fact that any strain produces only one of these 50 polysaccharides would lead one to suppose that the same gene is involved in all 50 cases and that there are more than 50 cytoplasmic materials which the gene can act upon. As will be brought out below in the section on mutations, the gene *K* in Paramecium also appears capable of controlling the production of either of at least two somewhat diverse cytoplasmic substances.

Another class of possibly similar cases is the class of hereditary bacterial adaptations.[6,7] A cell which is at first unable to produce a certain substance acquires the power to do so after subjection to an appropriate environment. In some instances the environment seems to act merely by favoring the growth of mutants which arise quite independently of the selective environment. It would appear worth while to investigate also the possibility that the hereditary properties of a strain could be altered by supplying the strain for a limited time with a small amount of the substance, or by extracts of strains that can produce it. Results of this general type have indeed been reported. *Eberthella typhosa*[8] acquires the ability, previously lacking, of synthesizing tryptophane after growth in a trytophane medium. *Proprionibacterium pentasaceum*[9]

similarly acquires the ability to synthesize vitamin B_1 after growth in its presence. Superficially, the parallel to Paramecium suggests a similar genetic interpretation.

3. Mutations.—The observations on Paramecium show that mutations, in the sense of hereditary changes of characters, may have physical bases not at present recognized. According to prevailing views, the physical basis of a mutation is either a change in the gene or in the number and arrangement of the chromosomes and their parts. The hereditary change from sensitive to killer, however, can occur without any change in the gene, merely by addition of the essential cytoplasmic substance to the cell with the killer gene. The reverse hereditary change from killer to sensitive can occur simply by the loss of this cytoplasmic substance. Still another class of hereditary changes has been observed in Paramecium: strong killers which act quickly on sensitive cells have changed to weak killers which act slowly. The data indicate that this change is due to a change in the cytoplasmic factor, not to a change in the gene. In addition to these three previously unrecognized kinds of physical basis for a mutation, mutations of the well-known type have also been observed. The killer gene itself may mutate to the sensitive allele. The mutated gene is unable to control the further production of the cytoplasmic material even when some of it is present.

In a system of determination and inheritance such as prevails in Paramecium, a number of relations concerning relative mutation rates might be predicted. Two of these will be briefly indicated. First, the rate of mutation in opposite directions should as a rule be very unequal. For example, mutations from killer to sensitive would be expected more frequently than in the reverse direction. This follows from the fact that mutations from killer to sensitive will occur either if the cytoplasmic factor is lost or if the gene mutates to a form that cannot control production of the cytoplasmic factor; whereas mutation from sensitive to killer, in those cases in which the sensitive gene is present, requires both mutation of the gene and *de novo* origin of the cytoplasmic factor. Thus mutation in one direction follows either of two events while mutation in the reverse direction under certain conditions requires both of two events. These two events need not occur simultaneously, but the genic change must precede the cytoplasmic one.

Second, the observed mutation rate could, under certain conditions, be dependent upon whether the gene was in homozygous or heterozygous condition. To illustrate, in organisms homozygous for the sensitive allele, mutation of this gene to the killer allele would ordinarily be observed only if the cytoplasmic factor also arose subsequent to the gene mutation; but in organisms heterozygous for these genes and containing the cytoplasmic factor, every mutation of k to K would be directly de-

tected at autogamy, for the cytoplasmic factor is already present and being maintained. The result of this situation is to give under certain conditions higher observed mutation rates in heterozygotes than in homozygotes. This would be expected only for the mutation of genes that do not control a cytoplasmic factor into alleles that do.

4. Development.[10]—The fact that the determiners of the phenotype may lie outside of the nucleus and still be under genic control, as shown in the case of the determination of the killer phenotype, appears to have particularly important bearings on the prime problem of development: the production of different characteristics in cells with the same genes. With the occurrence of such determiners in the cytoplasm and, at least at certain stages, not in the nucleus, all that is required to account for the production of different characters in different cells with the same genes is to have differential segregation of these cytoplasmic determiners at cell division, a condition which has long been known to occur for a number of visibly different cytoplasmic components. The fact that the genes are unable to control the production of the substances once they are removed from the cell will prevent the cells which lose the cytoplasmic factors from developing the same characters as those cells which retain them.

Further pursuit of the possible implications of the system of determination and inheritance in *P. aurelia* seems at present less urgent than attempts to see whether comparable phenomena occur in higher organisms. From the discussion above on the problem of cytoplasmic inheritance in higher plants, one might expect these to provide the most favorable material for such study.

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[2] Aided by a grant from the Rockefeller Foundation.

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[10] I am indebted to Dr. Boris Ephrussi for suggesting that the results on Paramecium might have bearings on problems of development.

THE RELATION BETWEEN NUMBER OF NUCLEOLI AND NUMBER OF CHROMOSOME SETS IN ANIMAL CELLS

BY G. FANKHAUSER AND R. R. HUMPHREY

DEPARTMENT OF BIOLOGY, PRINCETON UNIVERSITY, AND DEPARTMENT OF ANATOMY, SCHOOL OF MEDICINE, UNIVERSITY OF BUFFALO

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The relation between the number of nucleoli visible in telophase or interphase nuclei and the number of haploid chromosome sets which are present has been studied extensively in plants (cf. the recent review by Gates[1]). DeMol[2] first noted that diploid hyacinths have a maximum of two nucleoli, triploid three, and hypotetraploid four. This relationship became understandable when it was shown conclusively by Heitz[3] that, during telophase, the nucleoli are formed at specific points on specific chromosomes, usually at secondary constrictions which either mark off a satellite or are located some distance from the end of the chromosome. Since as a rule, each haploid set of chromosomes includes a single chromosome with a "nucleolar organizer," the normal diploid nucleus contains two nucleoli. The number of nucleoli may therefore be used as a criterion for the identification of polyploid individuals or races within a species and, with certain restrictions, for the determination of phylogenetic relationships between different plant species.

The relation of nucleolar number to polyploidy has received much less attention from zoölogists, largely because, until recently, there was little interest in the study of polyploidy in animals in its various aspects. This has led Gates[4] to the conclusion that "it is still uncertain in how far the number of nucleoli in animals can be used as an index of the number of sets of chromosomes." However, what evidence there is available in the zoölogical literature clearly points to the existence of the same constant relationship as obtains in plants, at least in ordinary somatic cells. Under special physiological conditions, however, (e.g., in gland cells and in growing oöcytes), the number of nucleoli may be greatly increased, apparently without a corresponding increase in chromosome number. The pertinent information on nucleolar number and heteroploidy will be reviewed briefly in this paper, and new evidence will be added from a study of the nucleoli in an extensive heteroploid series of larvae of the axolotl, *Amblystoma mexicanum*, and of larvae of mixed tigrinum-mexicanum ancestry.

That each haploid set of chromosomes may be associated with a single nucleolus was first indicated by Conklin's classical observations on the fertilization and cleavage of eggs of the gastropod Crepidula.[5] Each of the germ nuclei, before their union, contains a single nucleolus, while in the telophase of all the cleavage mitoses two nucleoli appear which may

fuse into a single one if the resting period is prolonged. The cleavage nuclei are clearly dual, and each half-nucleus, which is descended from one of the germ nuclei, contains a single nucleolus.

Direct evidence for a constant relationship between number of nucleoli and chromosome number was found by Parmenter[6] in his work on parthenogenesis in frogs. Epithelial nuclei of diploid tadpoles of *Rana pipiens* contain two nucleoli in a large percentage of the cells, and one in the remainder; haploid parthenogenetic tadpoles show only one nucleolus in all nuclei. In 1933 Parmenter extended his observations to a triploid and a mixed diploid-triploid larva of *Rana pipiens*, and a mixed diploid-triploid-tetraploid larva of *Rana palustris*.[7] The triploid nuclei contained three nucleoli, the tetraploid four. The same relationship obtained in haploid, diploid and triploid cells of parthenogenetic individuals of *Rana fusca*.[8]

Corresponding figures were published by Porter[9] for haploid, diploid and tetraploid cells of androgenetic larvae of *Rana pipiens*. A very extensive series of polyploid parthenogenetic larvae of *Rana nigromaculata* was investigated by Kawamura[10] who found that most of the nuclei in triploid larvae contained three nucleoli, in tetraploid larvae four and in hexaploid larvae five or six.

The number of nucleoli was also recorded in germ cells of polyploid silkworms by Kawaguchi.[11] Diploid, triploid and tetraploid spermatogonia showed two, three and four nucleoli, respectively. Diploid oögonia contained a single nucleolus, triploid and tetraploid oögonia two, i.e., the number of nucleoli corresponded to the number of *Z*-chromosomes present, indicating that in this species the *Z*-chromosome carries the nucleolar organizer.

Since the number of nucleoli, together with measurements of the nuclear size, may give a reliable estimate of the degree of polyploidy of nondividing cells (cf. Berger[12]) it promises to be particularly helpful in the study of partially polyploid organs or tissues in otherwise diploid individuals. In the normal liver of the rat, tetraploid and octoploid cells are abundant and may outnumber the diploid (Beams and King,[13] Sulkin[14]). In the mouse liver, the nuclei fall into three main size classes whose volumes are approximately as 1:2:4 (Jacobj[15]), indicating that they are diploid, tetraploid and octoploid, respectively. Biesele, Poyner and Painter[16] have shown that these nuclei contain a maximum of four, eight and sixteen nucleoli. A few very large nuclei, presumably 16-ploid, showed up to thirty-two nucleoli. Each chromosome set of the mouse apparently contains two nucleolar organizers, a fact which might suggest that this species is tetraploid. However, some species of plants, which are unquestionably diploid, also possess four nucleolar organizers.

The numerous scattered observations on polyploid tumor cells have

been reviewed in the same paper.[16] In their own investigations of the nuclear phenomena in mouse cancers these authors found evidence that the increased size of the cancer nuclei is a result of endomitosis which may lead to the formation of larger chromosomes with two, four, eight times, etc., the normal number of chromatids ("polytene chromosomes"), often combined with an increase in the number of separate chromosomes to the tetraploid, octaploid and higher levels. The actual valence of the nucleus, i.e., the total number of chromatids contained in it, is indicated by the number of nucleoli, although there is a strong tendency for the nucleoli to fuse, particularly in nuclei with polytene chromosomes. However, careful measurements of the volume of the nucleoli may still reveal their compound nature.

In the frequently highly polyploid somatic cells of water striders, fusion of two or more nucleoli seems to be the rule.[17] Diploid and tetraploid nuclei usually show one nucleolus, rarely two. In more highly polyploid nuclei, the number of nucleoli increases but remains far below the number of chromosome sets which may be accurately determined by counting the heterochromatic *X*-chromosomes.

Prolonged studies of spontaneous and induced polyploidy in several species of salamanders have not produced information on the behavior of the nucleoli until recently. In the three species which were studied first, *Triturus viridescens*, *T. pyrrhogaster* and *Eurycea bislineata*, the chromosome number of living larvae may be determined with ease in whole-mounts of amputated tailtips where the epidermis of the transparent fin offers excellent material for the study of mitotic figures.[18] The interphase nuclei, however, do not show distinct nucleoli following fixation in Bouin's and staining with Harris' acid haemalum, the technique which was found to be most satisfactory for the rapid preparation of the tailtips for chromosome counts. Last year, the investigations were extended to larvae of the axolotl, *Amblystoma mexicanum*.[19] In this species and in mexicanum-tigrinum hybrids, a considerable number of the tailtip preparations show the nucleoli clearly in some interphase nuclei while in other nuclei they are not easily distinguished from the numerous chromocenters; frequently also the structure of the nucleus is partly obscured by pigment granules in the cytoplasm.

Sufficient observations have been made to show a good agreement between the maximum number of nucleoli visible and the number of chromosome sets present, as determined by chromosome counts in the same tailtip. The series includes haploid, diploid, triploid, tetraploid and pentaploid larvae (table 1). Some of these represent spontaneous aberrations in chromosome number, but the majority developed from eggs that were refrigerated immediately after laying. Haploid nuclei always contain a single nucleolus (Figs. 1 and 2), diploid nuclei two in the great majority

of cells, while in the remaining cells the nuclei contain one whose size may reveal its origin through fusion of two separate nucleoli (Figs. 3 and 4). Triploid individuals show three nucleoli in most of the cells, two in the remainder (Fig. 5); tetraploid nuclei have four nucleoli in over one-half of the cells, and three (rarely two), in the others (Fig. 6). In the tailtip of a pentaploid larva five nucleoli were found in a majority of the cells, while most of the remaining nuclei contained four (Fig. 7).

The demonstration of this simple numerical relationship in epidermis cells of axolotl larvae is particularly interesting in view of the fact that Dearing[20] described the formation of the nucleoli during telophase at the secondary constriction of a particular pair of chromosomes in epithelial cells of larvae of *Amblystoma tigrinum*. These observations, together with those of Kaufmann[21] on ganglion cells of *Drosophila larvae*, were the first

TABLE 1

CHROMOSOME NUMBER, NUMBER OF NUCLEOLI AND NUCLEAR SIZE IN EPIDERMIS CELLS OF TAILTIPS OF AXOLOTL LARVAE

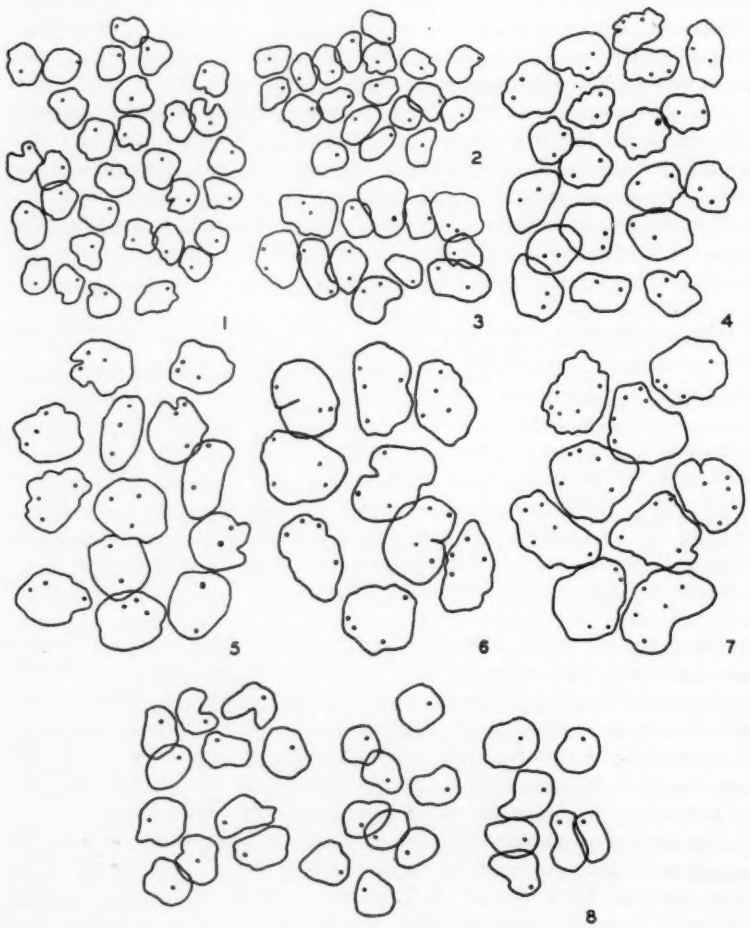
	HAPLOID	DIPLOID	TRIPLOID	TETRAPLOID	PENTAPLOID
Chromosome number	14	28	42	56	70
Maximum number of nucleoli	1	2	3	4	5
Area of 20 nuclei (×630) in square inches:					
No. of tailtips	14	100	96	2	2
Range	1.87–2.47	3.30–5.80	5.27–9.25	8.50–8.74	8.05–10.65
Average	2.23	4.37	6.67	8.62	9.35
Ratio	1.00	1.96	2.99	3.86	4.19

to show the existence of nucleolar organizers in animal cells. Dearing also found that the vast majority of epithelial cells contained two nucleoli, while the large single nucleolus visible in some cells was clearly a compound structure formed by the fusion of the two nucleoli during telophase. A single larva in his material, although unquestionably diploid, showed three nucleoli in each nucleus, the third nucleolus being associated with a chromosome that normally did not possess a nucleolus organizer.

Dearing's observations also offer a simple explanation for an apparent exception to the rule found among our axolotl larvae. The tailtip of one very abnormal larva showed epithelial nuclei within the diploid size range but with a single nucleolus (Fig. 8). In several mitotic figures from different regions of the tail fin the chromosome number was established as twenty-four, i.e., $2N - 4$. Obviously, one of the nucleolus-forming chromosomes was missing in this hypodiploid animal.

The availability of an additional criterion for the determination of the degree of polyploidy is particularly valuable in the axolotl in which accurate chromosome counts are often difficult to make because of the

scarcity of clear metaphase figures; as a result, identification of polyploid individuals was frequently based on measurements of nuclear size alone. As is seen from table 1, the area of camera lucida drawings of twenty



FIGURES 1–8
(For explanation, see opposite page)

epidermis nuclei is almost directly proportional to the chromosome number, except for the pentaploid nuclei in which the increase is relatively too small. However, only two pentaploid individuals were available; in

view of the considerable range of variation between individual tailtips it may be expected that measurements on more pentaploids will raise the average.

It should be pointed out that the epidermis nuclei of the tail fin are thin discs whose thickness increases very slightly with the chromosome number so that the area measured in the drawings is closely representative of the nuclear volume. This observation has a more general interest since it indicates that the thickness of the epithelium is kept more or less constant, regardless of the size of the individual cells which compose it. The cells are flattened to a greater or lesser extent to fit this condition. Preliminary observations on sections of various polyploid individuals have shown that a similar adjustment takes place in other thin layers of cells.

Summary.—(1) A review of the few reported observations on the number of nucleoli in nuclei of haploid and polyploid animal cells shows that the same relationship obtains as in plants, i.e., the nucleolar number increases in direct proportion to the chromosome number. In spermatogonia of polyploid silkworms and in epithelial cells of frog tadpoles the nuclei contain one nucleolus for each chromosome set. In normal liver cells and in cancer cells of the mouse the nuclei contain a maximum of two nucleoli for each chromosome set, although the great tendency of the nucleoli to fuse may obscure this relationship. In polyploid tissues of diploid water striders, fusion of the nucleoli occurs so generally that the nucleolar number is of little value in the estimation of chromosome numbers.

(2) New observations demonstrate a similar relationship in epithelial

EXPLANATION OF FIGURES

All figures are camera lucida drawings of epidermis nuclei from the tail fin of young axolotl larvae. The original magnification was ×630; it was reduced to ×270 in reproduction.

1. From a haploid larva ($N = 14$). All nuclei have a single nucleolus.
2. From the left (haploid) side of the tailtip of a haploid-diploid mosaic larva.
3. From the right (predominantly diploid) side of the same mosaic larva. A few haploid nuclei are present. The diploid nuclei have mostly two nucleoli; one nucleus contains a single large fusion nucleolus.
4. From a diploid larva. All nuclei but one with two nucleoli.
5. From a triploid larva. Of the thirteen nuclei shown, nine have three nucleoli, four have two.
6. From a tetraploid larva. Seven of the nine nuclei shown have four nucleoli, the other two have three.
7. From a pentaploid larva. Six of the nine nuclei shown have five nucleoli, three have four.
8. From a hypodiploid larva with twenty-four chromosomes ($2N$-4). Three groups of nuclei from different regions of the tail fin are drawn to show that all nuclei contain a single nucleolus; presumably, one member of the pair of chromosomes with nucleolar organizers is missing.

cells of heteroploid axolotl larvae, covering the whole range from haploidy to pentaploidy (Figs. 1 to 7, table 1). The maximum number of nucleoli visible in the nuclei of any individual corresponds to the number of haploid chromosome sets. A single larva with nuclei within the diploid size range showed a single nucleolus in all nuclei (Fig. 8). This animal was found to be hypodiploid ($2N - 4$); presumably, one of the missing chromosomes contained a nucleolus organizer.

(3) The available evidence supports the conclusion that the number of nucleoli may be used as a criterion in the diagnosis of completely polyploid individuals or of polyploid cells or tissues in animals as well as in plants.

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ON PRIMARY ATTRIBUTES OF ALLELES IN DROSOPHILA MELANOGASTER

BY CURT STERN AND ELIZABETH WHITE SCHAEFFER

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF ROCHESTER[1]

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The mutant gene *cubitus interruptus* (*ci*), in the fourth chromosome of *Drosophila melanogaster*, causes a long gap in the fourth wing vein if it is present in hemizygous condition. If two, instead of one, alleles are present, *ci*/*ci*, the gap is shorter, and with three alleles, in the triplo-IV genotype, *ci*/*ci*/*ci*, often no gap appears[2] (Fig. 1). In contrast to this cumulative ef-

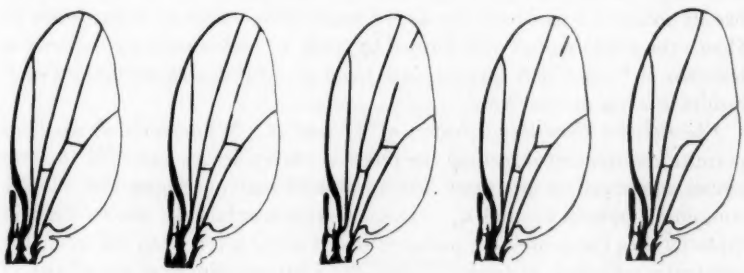

FIGURE 1

Classification of interruptions of cubital veins (for details see[2]).

fect of increased doses of *ci* is an antagonistic effect found when *ci* is combined with certain normal alleles of the *ci* locus.[2, 3] Thus, the allele $+^3$ in hemizygous state produces, nearly always, normal venation, while flies with $+^3$ in combination with *ci*, $+^3/ci$, often have interrupted veins. To explain these and related results, the concept has been developed that a gene, in producing its ultimate phenotypic effect, acts at first upon a cellular substrate, *S*, and changes it into a product, *P*, which functions in the elaboration of a normal phenotype. It was further postulated that the action of a gene upon its substrate involves at least two factors: The "combining power," *c*, of the gene, as measured by the amount of *S* with which it is able to interact when *S* is in excess, and the "efficiency," *e*, with which the gene converts *S* into *P*. The operation of this system of primary gene action may be seen in its application to the apparently contradictory cumulative and antagonistic effects of *ci* on wing venation described above: In the former case it is assumed that *S* is present in excess of the combining power of the *ci* genes, but that one *ci* by itself is not able

to manufacture nearly enough P to give a normal wing. Doubling c by adding another *ci* or tripling by adding two *ci* brings the wing progressively closer to normal, as would be expected. The cumulative effect (toward normality) of additions of *ci* genes is thus due to the increased total amount of P produced, each *ci* gene combining with a fraction of S and contributing its increment of P.

The second case, in which *ci*, in combination with $+^3$, appears to decrease, rather than increase the approximation to the normal vein condition, can be explained if it is assumed that the efficiency of *ci* is less than that of $+^3$, and that the amount of S available is less than the maximum with which $+^3$ and *ci* together can combine. The alleles $+^3$ and *ci* are then competing for a limited S, so that they deprive each other of their full share of S. Since the efficiency of *ci* is less than that of $+^3$, *ci* does not utilize its share of S as effectively as $+^3$ would have—that is, it forms less P. Hence the total amount of P formed by both $+^3$ and *ci* working together is less than $+^3$ could have formed alone from S. This decreased amount of P results in a less normal wing.

The Relative Combining Powers of $+^3$ *and ci.*—While such an analysis permits a statement regarding the relative efficiencies e_{+^3} and e_{ci} of the two alleles concerned, it does not lead to deductions concerning the relative combining powers c_{+^3} and c_{ci}. Dosage experiments have shown that in diplo-IV flies the combining power of one *ci* allele is less than the available substrate, and that, in triplo-IV flies, the joint combining power of two *ci* alleles is less than the substrate available in such a constitution. Though it would greatly simplify the analysis if it could be assumed that the amount of substrate is alike in diplo-IV and triplo-IV flies, no relevant data are available, and the two constitutions must be treated separately. Furthermore, in neither case is it known how much of the substrate is claimed by a $+^3$ allele.

An estimate of the relative combining powers of $+^3$ and *ci* was made possible by an experiment in which the phenotype of triplo-IV flies of the constitution $+^3/ci/M$-4 was determined (table 1[4]). It is seen that 56 out of 158 tested males with abnormal venation were of the constitution $+^3/ci/$-M-4 (in addition to an unknown number among the normal overlaps not tested). Since nearly all $+^3/M$-4 flies are normal, and since the addition of an M-4 chromosome to various genotypes has been shown to result in phenotypic shift toward normality,[2] $+^3/M$-4/M-4 can be likewise regarded as normal. A comparison of the phenotypes of $+^3/ci/M$-4 (less than normal) and $+^3/M$-4/M-4 (normal) thus indicates that competition for a limited substrate occurs between $+^3$ and *ci* in $+^3/ci/M$-4. In other words, the joint combining power of the two different alleles c_{+^3} and c_{ci} is larger than S. It is known, on the other hand, that the phenotype of $ci/ci/M$-4 is less normal than that of *ci/ci/ci* which means that the joint combining

powers of two *ci* alleles do not exhaust the substrate. In summary, $P(+^3/ci/M\text{-}4) < P(+^3/M\text{-}4/M\text{-}4)$, and therefore

$$c_{+^3} + c_{ci} > S, \tag{1}$$

and since $P(ci/ci/M\text{-}4) < P(ci/ci/ci)$, and therefore

$$c_{ci} + c_{ci} < S, \tag{2}$$

it follows, from (1) and (2), that

$$c_{+^3} > c_{ci}. \tag{3}$$

Relation (3) completes the description of the two alleles $+^3$ and *ci* in terms of both relative combining and efficiency factors. The normal allele possesses a greater combining ability and a greater efficiency than the mutant allele.

TABLE 1

(*a*) Not-Minute F_1 ♂♂ of $+^3/+^3 \times ci\ ey^R/ci\ ey^R/M\text{-}4$,

	PHENOTYPIC CLASSES*			
N	0	1	2	3
134	53	100	14	..

(*b*) Of the above F_1 ♂♂ tested for being $+^3/ci\ ey^R/M\text{-}4$, by mating to unrelated females

0	1	2
47	99	12

(*c*) Of these the following were $+^3/ci\ ey^R/M\text{-}4$ as shown by appearance of at least three or in a few cases of two *M*-4 F_1 flies

0	1	2
21	32	3

* *N* = uninterrupted distal section of 4th vein; 0 = uninterrupted but thinned section; 1, 2 = different progressive degrees of interruption; 3 = absence of whole section.

TABLE 2

	♀♀					♂♂				
	N	0	1	2	3	*N*	0	1	2	3
ci^W/ci^W	..	.	.	.	892	..	.	.	1	891
$ci^W/ci\ ey^R$*	..	.	.	4	980	..	.	.	6	959
$ci^W/M\text{-}4$*	4†	.	.	.	...	3†	.	.	1	1

* These two groups of flies were derived from the same parents from crosses of $ci^W/ci^W \times ci\ ey^R/M\text{-}4$.

† Some of these may have been of Class 0.

The Relative Efficiencies of ci^W and other ci Alleles.—A similar analysis is possible for another mutant allele of the *ci* locus, ci^W. While *ci* in general acts as a recessive in combination with a wild allele (the allele $+^3$ being an exception to this statement) ci^W is dominant in heterozygous combination with any one of the three known normal alleles of the *ci* locus.[3] Homozy-

gous ci^W/ci^W causes a very striking reduction of the venation, placing nearly all individuals into the most extreme Class 3 (table 2; all experiments referred to hereafter were made with stocks isogenic for all chromosomes except the fourth). Heterozygotes with normal alleles show wide variability with many individuals completely normal phenotypically (see table 5[3]). Heterozygotes of ci^W with the "recessive" ci are very similar to ci^W/ci^W homozygotes but seem to contain a slightly larger number of the less extreme Class 2 individuals (table 2). These facts permit certain deductions: Since, at 26°, the average phenotypes of heterozygotes of ci^W and any of the three normal alleles are more extreme than hemizygotes of any of the normal alleles, which practically always appear normal, it follows that

$$e_{ci^W} < e_{+^c}, \text{ or } e_{+^3}, \text{ or } e_{+^4}. \tag{4}$$

Since it is further true that ci^W/ci is more extreme than ci/M-4, which has been shown to have its phenotypic mode in Class 2[2], it follows that

$$e_{ci^W} < e_{ci}. \tag{5}$$

This result can also be deduced from another experiment. In a cross of $ci^W/ci^W \times ci/ci/M$-4 the three genotypes ci^W/ci, $ci^W/ci/ci$, and $ci^W/ci/M$-4 were distributed among Classes 1 to 3 (table 3, *a*). Since it was shown that

TABLE 3

(*a*) Not-Minute F_1 ♂♂ of $ci^W/ci^W \times ci\ ey^R/ci\ ey^R/M$-4)

N	0	1	2	3
.	.	3	78	671

(*b*) Of the above F_1 ♂♂ tested by crossing to unrelated females, for being $ci^W/ci\ ey^R/$-*M*-4 (flies hatched during the first four days in four out of six cultures)

1	2	3
1	23	182

(*c*) Of these the following were $ci^W/ci\ ey^R/M$-4*

1	2	3
.	1	75

* The appearance of at least three or in a few cases of two Minute flies was considered proof of the paternal constitution as $ci^W/ci\ ey^R/M$-4. The one P ♂ of Class 2 and two males in Class 3 gave rise to only one *M* fly in F_1. These three *M* flies may not have been *M*-4 but newly arisen mutants.

only very few ci^W/ci flies belong to Class 2 and since progeny tests (table 3, *b*, *c*) prove that the same is true for $ci^W/ci/M$-4 flies, most of the individuals of Class 2 must have been of the constitution $ci^W/ci/ci$. Furthermore, it is known that the average phenotype of M-4/ci/ci flies is considerably more normal than Class 2. Therefore, the "addition" of ci^W to the latter genotype, transforming it to $ci^W/ci/ci$, results in a decrease of normality, i.e.,

in competition for substrate between ci^W and ci with $e_{ciW} < e_{ci}$. It may be added that this relation not only extends the result obtained in expression (4) but also confirms it *a fortiori* since it is known that

$$e_{ci} < e_{+c}, \text{ or } e_{+3}, \text{ or } e_{+s}.$$

The Relative Combining Powers of ci^W and ci.—In order to obtain an estimate of the combining power of ci^W we employ reasoning similar to that used in comparing c_{ci} and c_{+s}. The phenotypes of flies of the constitution $ci/M\text{-}4/M\text{-}4$ were compared with those of the type $ci/ci^W/M\text{-}4$. The latter were found to be nearly exclusively of the most extreme Class 3 (table 3, *b*, *c*) while the former are known to be generally more normal. This means that the "addition" of ci^W to $ci/M\text{-}4/M\text{-}4$ results in a decrease in *P*, an indication of competition between *ci* and ci^W. Since, as stated before, it is known that two *ci* alleles do not exhaust the substrate available in a triplo-IV fly, whereas competition of *ci* and ci^W indicates limitation of substrate relative to the combining powers of these two alleles, it follows that

$$c_{ciW} > c_{ci}. \qquad (6)$$

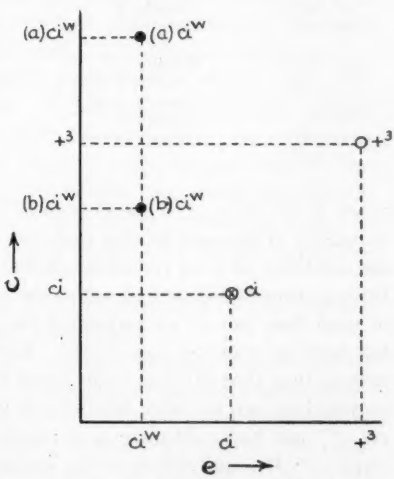

FIGURE 2

Diagram of possible relations of combining and efficiency factors of the alleles $+^3$, *ci* and ci^W. The seriation of the three *e* factors is established but not their relative distances. The seriation of the *c* factors is only established in so far as $c_{+3} > c_{ci}$ and $c_{ciW} > c_{ci}$; therefore two possiblities for c_{ciW} are indicated: (*a*) $c_{ciW} > c_{+3}$, and (*b*) $c_{ciW} < c_{+3}$.

The expressions (5) and (6) signify an interesting difference between the two mutant alleles *ci* and ci^W: the combining power of ci^W is larger than that of *ci*, but its efficiency is smaller. No data are available at present which relate the combining power of ci^W to that of a normal allele. The relations of the three alleles $+^3$, *ci* and ci^W are illustrated in figure 2.

The Multiple Effects of ci^W and Its Primary Action (Table 4).—A very peculiar effect was observed when attempts were made to obtain flies hemizygous for ci^W. It was found that the genotype $ci^W/M\text{-}4$ is nearly fully lethal, with flies completing their pupal development but unable to emerge. Hemizygotes dissected from pupae invariably showed shortened, swollen,

crippled legs so that their inability to hatch is obviously due to a great exaggeration of the slight crippling of legs occasionally found in homozygous ci^W/ci^W. Another characteristic exaggerated in ci^W/M-4 flies is the presence of extra bristles on the thorax, a feature found only rarely in our isogenic

TABLE 4

THE MULTIPLE EFFECTS OF *ci* ALLELES (FURTHER DETAILS IN TEXT)

GENOTYPE	VENATION CLASS	LEGS	EXTRA BRISTLES
ci^W/M-4	Usually *N*	Crippled	Few
ci^W/ci^W	Usually 3	Very slightly crippled	Very rare
ci^W/ci	Usually 3	Normal	None
$ci^W/+$	Usually 1 or 2	Normal	None
ci/ci	Usually 1 or 2	Normal	None
$ci^D/+$	Usually 3	Normal	None
ci^D/ci	Usually 3	Usually normal	Many
ci^D/ci^W	3	Crippled	Very many

stock of ci^W/ci^W. A single dose of ci^W is thus less effective in making for normality of legs and bristles than two doses. In contrast to this finding is the condition of wing venation. While it was not possible to classify wings from mature flies dissected out of the pupae, this was done with the wings of nine flies, out of an estimated two thousand, which had succeeded in hatching more or less completely. Eight of these showed a venation more normal than that of Class 3 (the ninth fly was of Class 3 phenotype, but had normal legs and no extra bristles. It was probably not ci^W/M-4 but rather ci^W/ci^W and had a Minute appearance due to a new mutation.). Of the eight ci^W/M-4 individuals seven seemed to have normal wings though it could not be excluded with certainty that some were of the slightly abnormal Class 0. The eighth fly was of Class 2 (table 5).

TABLE 5

	PHENOTYPES OF ci^W/M-4			
	*N**	0	1	2
Mature flies	7	.	.	1
Pupal wings	17	5	5	5
Total		29	5	6

* Some of these may have been Class 0.

The possibility was considered that the eight individuals which had succeeded in hatching might have been a selected group with unusual normality of wing venation. Accordingly, the phenotype of early pupal stages—before any selective influences would presumably have entered in—was ascertained. As Waddington (1940[5]) has shown, the typical venation of *ci* is visible in individuals of less than two days' pupal age. We found that the crippled legs are also clearly differentiated from normal legs at this time so that dissections of pupae from the cross $ci^W/ci^W \times M$-4/*ci* enabled

us to ascertain the constitution as ci^W/M-4(crippled) or ci^W/ci (normal legs). Thirty-two wings, from 19 individuals of the genotype ci^W/M-4, were fixed and stained in Delafield's hematoxylin and mounted in Canada balsam (table 5). Twenty-two wings were either completely normal, or of Class 0. In ten cases, the wings belonged to Classes 1 and 2. The controls, ci^W/ci, were all of Class 3 (13 specimens). There is thus no doubt that ci^W/M-4 (one ci^W) are more normal in venation than their ci^W/ci^W sibs (two ci^W).

A still more remarkable fact is that the wing phenotype of ci^W/M-4 hemizygotes is even more normal than that of $ci^W/+$ heterozygotes which have their mode in Classes 1 or 2^3 (see particularly the heterozygote $ci^W/+^3$). How unusual this relation is will be realized most clearly when it is pointed out that both the $+^3$ and the ci^W hemizygotes make for largely normal wings but that the heterozygote between these two alleles is strongly abnormal. Except for some analogy with Hinton's (1942[6]) incompletely analyzed Ey^{D39k} allele in Drosophila the only comparable though not identical case known to us is that of the various sex alleles in Habrobracon where the hemizygotes are all males while the heterozygotes are females (Whiting, 1940[7]).

An attempt to incorporate these data into the concept of genic action referred to earlier has led to the following considerations:

On the basis of the dosage data from *ci* and its + alleles it was postulated that the effect of all *ci* alleles in regard to venation is fundamentally identical, the variations from one allele to another consisting only of differences of the combining power with the substrate and of the efficiency in transforming the substrate. Under this assumption the effect of the venation reaction of different *ci* alleles may vary from zero, in case the *c* or *e* factor, or both, are zero, to various positive values. A negative value for the product of the gene-substrate interaction would be impossible. Just such a negative value, however, is demanded by the observation that two ci^W alleles cause less normal venation than one ci^W allele. Therefore, an additional hypothesis is required if this case is to conform with the postulate that all *ci* alleles act basically in an identical way on venation. Two possibilities suggest themselves: (1) It is conceivable that another locus, or other loci, besides *ci*, combine with the substrate *S* and transform it into a product (*P*) effective toward normal wing venation. (The existence of the "other" locus could be visualized as due to a "repeat" of the *ci* locus.) If *S* is limited, competition between this other locus and ci^W would result, and if the efficiency of ci^W is lower than that of the other locus, the overall effect toward normal venation would be less in the presence of two ci^W alleles than of one. (2) As an alternative explanation of the observed effect of the ci^W allele, it might be assumed that the ci^W allele interacts with the same substrate *S* in two different ways, one leading toward normality of venation, the other toward an unrelated product. Under this assumption it can be seen

that competition for S between the two reactions might occur in such a way that one ci^W allele obtains sufficient substrate for the venation reaction to lead to near-normality, while two ci^W alleles, or the two alleles $ci^W/+$ combined, are not successful in this respect. This hypothesis implies that each of the combining properties of the two reactions are not simply proportional to the number of ci^W alleles but approach saturation values with different velocities. If this were not so, the shares of the substrate which the wing and the other reaction obtain would remain the same in ci^W/M-4 and ci^W/ci^W.

The hypothesis of two reactions of the ci_W allele competing for the same substrate may be fitted specifically to the leg or the bristle effect of ci^W. If it is the reaction toward normal leg or bristle development which competes with that for wing development, the correlation of crippled legs or extra bristles with normal wing in ci^W and of normal legs or normal bristles with deficient wing in ci^W/ci^W becomes understandable. Viewed from this angle the low efficiency of ci^W in terms of wing development would be due to the competing leg or bristle reaction whose efficiency in terms of normal venation may be zero.

It might be questioned whether the leg, bristle and wing effects are expressions of one and the same allele ci^W or rather stem from other loci in the fourth chromosome. The latter alternative is improbable since all these effects presumably arose simultaneously by mutation. In addition, the bristle effect exists in other alleles of the *ci* locus. While neither ci/ci nor $ci^D/+$(ci^D is a dominant allele, lethal homozygously) show extra bristles typically, the heterozygote ci^D/ci has very many (Dr. Gertrude Heidenthal, unpubl.; confirmed by the present authors). The legs of ci^D/ci are only slightly abnormal, if at all, the sexcombs are large, resembling those characteristic for some ci^W stocks. The compound ci^D/ci^W is similar to the ci^W/M-4 hemizygote in having extremely crippled legs, which leaves it unable to emerge from the pupa case. The number of extra bristles is still larger than in ci^D/ci flies. Wings from seven individuals could be classified; they belonged to the extreme Class 3.

Whichever of the two explanations proposed for the venation effect of different doses of ci^W may approximate the truth, a single primary action of the ci^W allele seems ruled out by the facts: In order to allow for the cumulative effects toward normality of leg and bristle phenotype of two versus one dose of ci^W, a substrate must be *in excess* of the combining power of one ci^W allele, while in order to cause the decreased effect on venation of two versus one dose of ci^W, *limitation* of substrate relative to the combining power of one ci^W allele must exist. The apparent paradox implies at least two primary reactions of ci^W in which the relations of substrate to combining power differ. This difference may be due (*a*) to two basically different reactions of ci^W, either with two different substrates, or, if with one sub-

strate, with different combining powers. Or (*b*) the difference between wing and leg reactions may be due to two exactly identical reactions of ci^{W} with the same kind of substrate, *S*, which, however, take place in different cells during development, *S* being in excess of the combining power where leg formation is involved but *S* limited where venation is influenced. This possibility points to the need of further precision in the definitions of unitary vs. multiple primary gene action as discussed by various authors. Unitary primary gene action has been conceived as a single event in development from which one or more further events may follow while multiple primary gene action was implicitly regarded as signifying more than one basically different gene activity. It is, however, apparent that the same gene activity exercised at different places or times in development may find sufficiently different surroundings to result in very different effects. This possibility then traces multiple gene effects to multiple primary events though these events are of similar type. Such genic action may be called *repetitive*, to differentiate it from unitary as well as from multiple primary gene action in the older sense. Repetitive gene action may be due to prolonged, continuous or to recurrent gene activity.

Arguments related to the problems of primary gene action have been based on two different types of facts. In one, cases of multiple phenotypic effects of alleles were regarded by some as due to multiple primary action. Those opposed to this reasoning have pointed out that apparently unrelated phenotypic effects could be shown in several cases to depend on a single peculiar embryological mechanism ("spurious pleiotropism") and have argued that the principle of unity of gene action "may be adopted unless and until the occurrence of genuine pleiotropism can be conclusively demonstrated" (Grüneberg[8]). This viewpoint has not gone unchallenged (e.g., Dobzhansky[9]). Thus multiple phenotypic effects cannot be admitted as decisive evidence in regard to the type of primary gene action. The other kind of facts bearing on the problem of primary gene action is the existence of multiple alleles which can be arranged in two or more non-parallel series according to their effect on two or more different phenotypic characteristics. For example, among the R alleles in *Zea mays*, R^{rg} and r^{rg} both cause colored plant parts, the former allele giving colored, the latter colorless aleurone, while the alleles r^{gr} and R^{gr} both cause colorless plant parts but differ in that the former has colored, the latter colorless aleurone.[10] An interpretation of facts like these in terms of multiple primary gene action is suggestive, but it could also be considered that each of the different alleles causes basically different unitary primary reactions. While such an assumption may appear less likely than the opposite one based on the postulate of basically identical effects of alleles of one locus on a given character, it should be pointed out that the postulate must depend on specific evidence of the type provided by dosage experiments in which the effect of

only one kind of allele, in different amounts, is studied. Such evidence is lacking in most cases. It is available for the *ci* locus: The recognition of repetitive or multiple primary action of ci^W depends on different dosage effects of ci^W and other alleles of the *ci* locus on two different characters. An attempt to regard the multiple effects of ci^W as secondary ones, all depending on unitary primary gene action, would meet with the difficulty that the effect of different doses of the *ci* allele shows the whole range of wing phenotypes from the extreme Class 3 to normality without any leg or bristle effects. Their appearance in the case of ci^W seems due to primary properties which distinguish the alleles ci^W and *ci*. Whether the leg and bristle effects themselves are due to a single primary gene reaction or whether they again are due to separate repetitive or multiple primary reactions remains undecided. Regardless of the future of the specific hypotheses proposed, it appears that a two-dimensional concept of genic action, in the form of a single reaction with constant amount of substrate and a combining and an efficiency factor, while giving a satisfactory account of one of the multiple effects, is not sufficient to explain the observations concerning more than one effect. The experimental facts seem to demand the introduction of additional primary attributes of the gene, such as multiple or repetitive action.[11] The further problem as to whether any one of the primary reactions considered is really a single one or consists of a whole group of reactions is completely beyond reach at present.

Summary.—1. A comparison of the effects on wing venation of the normal allele $+^3$ and the mutant allele *ci* shows that the normal allele has both a greater combining power with the substrate and a greater efficiency than the mutant allele.

2. The combining ability of the mutant allele ci^W is larger than that of *ci*, while the efficiency of ci^W is smaller than that of *ci*.

3. Hemizygotes ci^W/M-4 possess mostly normal wings, but heavily crippled legs and extra bristles. Homozygotes ci^W/ci^W have abnormal wings, and mostly normal legs and bristles. Heterozygotes $ci^W/+^3$ are similar to, though less extreme than, ci^W/ci^W.

4. The bristle and leg characters of ci^W are presumably due to the ci^W locus and not to independent loci, since the compound between two other alleles, ci^D and *ci*, also shows marked excess of bristles and slight leg effects. The heterozygote ci^D/ci^W is similar, in bristle and leg abnormalities to ci^W/M-4.

5. An explanation of the action of ci^W in terms of a single primary reaction of this allele seems ruled out by the facts. It appears that either multiple, different, primary reactions of ci^W are involved or that a single reaction is repeated at different places or times in development causing different effects ("repetitive-action").

[1] Supported in part by a grant from the Rockefeller Foundation.

[2] Stern, C., *Genetics*, **28**, 441–475 (1943).

[3] Stern, C., and Schaeffer, E. W., *Proc. Nat. Acad. Sci.*, **29**, 361–367 (1943).

[4] The chromosome carrying *ci* also contained the eyeless allele ey^R. While useful as a marker, it has been shown to have no influence on the venation type. Its presence is indicated in the tables but not in the text.

[5] Waddington, C. H., *Jour. Genetics*, **41**, 75–139 (1940).

[6] Hinton, T., *Amer. Natur.*, **76**, 219–222 (1942).

[7] Whiting, P., *Jour. Morph.*, **66**, 323–355 (1940).

[8] Grüneberg, H., *Proc. Roy. Soc. B*, **125**, 123–144 (1938).

[9] Dobzhansky, Th., *Genetics*, **28**, 295–303 (1943).

[10] Emerson, R. A., Beadle, G. W., and Fraser, A. C., *Cornell Univ. Agric. Exp. Sta. Memoir*, **180** (1935).

[11] The case of the scute-alleles, which Tsubina (*Biol. Zh. Mosc.*, **4**, 1004 (1935)) has shown to act cumulatively toward normality for some bristles and in the opposite direction for other bristles, may find its explanation in terms of repetitive gene action in regions of different amounts of substrate.

ON WILD-TYPE ISO-ALLELES IN DROSOPHILA MELANOGASTER

BY CURT STERN AND ELIZABETH WHITE SCHAEFFER

DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF ROCHESTER[1]

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In experiments on the fourth chromosome locus, cubitus interruptus (*ci*), of *Drosophila melanogaster*, three homozygous stocks were obtained, which were isogenic except for the fourth chromosomes which came from three different sources. At the usual culturing temperature of 25–26°, all three stocks show normal wing venation and thus all seem to contain "the" normal allele of the *ci* locus. Tests reported below showed that each stock contains a different normal allele. Different alleles indistinguishable except by special tests will be called iso-alleles.

Origin of the Three Wild Iso-Alleles.—The first iso-allele occurs in the "Canton-Special" wild stock established by Bridges.[2] This allele is designated as $+^C$. Another wild iso-allele of *ci*, designated $+^2$, is in a fourth

TABLE 1

HOMOZYGOTES, 26°C. (UNLESS STATED OTHERWISE)

♀♀ + ♂♂	$+^C/+^C$	$+^2\,ey^2/+^2\,ey^2$	$+^2/+^2$
*N**	2786†	1958	2900

* Phenotypic classes: *N* = uninterrupted distal section of 4th vein; 0 = uninterrupted but thinned section; 1, 2 = different progressive degrees of interruption; 3 = absence of whole section.

† The majority of these flies was raised at 18°C.

chromosome carrying the eyeless mutant ey^2. The third iso-allele, $+^3$, was derived from a heterozygous culture of unclear origin. (The wild-type alleles of *ci* would ordinarily be designated $+^{ci-1}$, $+^{ci-2}$, etc., but since only this locus is referred to in this paper, the simpler symbols $+^c$, $+^2$, $+^3$ are employed throughout.) Table 1 records the numbers of homozygotes of these three alleles inspected at 26° or 18°. All were normal.

Tests of Homozygotes at Low Temperature (*Table 2*).—It is known that

TABLE 2

HOMOZYGOTES, 14°C.

	♀♀		♂♂	
	N	0	*N*	0
$+^c/+^c$	435 ♀♀ and ♂♂: all *N*			
$+^2\ ey^2/+^2\ ey^2$	455	..	510	..
$+^3/+^3$	918	24	801	21

low temperature accentuates the phenotypic effect of *ci*, whether produced by mutant alleles or a hemizygous wild allele.[2] If the three iso-alleles, in homozygous state, are raised at 14° ± 1° (with occasional greater fluctuation of temperature) two of them, $+^c$ and $+^2$, still remain exclusively within the normal phenotype, but the third, $+^3$, is shifted in expression so as to lead to a small number of individuals which are slightly *ci* type.

Tests of Hemizygotes (*Table 3*).—At 26° flies carrying any one of the iso-

TABLE 3

HEMIZYGOTES

	26°C.				14°C.					
	♀♀		♂♂		♀♀			♂♂		
	N	0	*N*	0	*N*	0	1	*N*	0	1
$+^c/M$-4	130	.	126	1	297	3	2	232	12	5
$+^2\ ey^2/M$-4	1008	.	1122	.	251	31	3	276	27	.
$+^3/M$-4	317	3	368	3	141	10	1	75	9	.

alleles and the fourth chromosome *M*-4, which is deficient for a section including the *ci* locus, show the normal phenotype nearly exclusively, though a few are of the slightly abnormal Class 0. At low temperature proportionately more flies show the thinning or interruption of the fourth vein typical of *ci*. Differences between the alleles, in distribution of phenotypes, can be seen in table 3. The hemizygotes for $+^2$ and $+^3$ have a larger proportion of *ci* phenotypes than those for $+^c$. No clear difference appears between $+^2/M$-4 and $+^3/M$-4 flies. Together, the two tests, low temperature with homozygosity, and low temperature with hemizygosity, separate the three iso-alleles, the former demonstrating that $+^3$ differs from both $+^c$ and $+^2$, and the latter that $+^c$ differs from both $+^2$ and $+^3$.

Tests of Heterozygotes with ci (*Table 4*).—Flies heterozygous for ci^3 raised

TABLE 4

HETEROZYGOTES WITH *ci ey*^{R}

	26°C. ♀♀				26°C. ♂♂				14°C. ♀♀				14°C. ♂♂			
	N	0	1	2	*N*	0	1	2	*N*	0	1	2	*N*	0	1	2
$+^{c}$ + *ey*/*ci ey*^{R}	1081	...	..	.	960	...	...	.	621	21	3	.	493	100	12	..
$+^{2}$ *ey*^{2}/*ci ey*^{R}	1273	...	..	.	1148	...	...	.	1043	14	...	.	863	42	1	..
$+^{3}$ + *ey*/*ci ey*^{R}	384	114	52	.	206	122	111	5	170	223	122	7	135	209	123	20

at 26° fall clearly into two groups: $+^{c}/ci$ and $+^{2}/ci$ are all normal, while $+^{3}/ci$ flies are distributed over the phenotypic range of Classes *N* to 2. At low temperature $+^{3}/ci$ flies are further shifted toward abnormal venation. In addition some of the heterozygotes for $+^{c}$ and $+^{2}$ exhibit *ci* venation. The data show a difference in the expression of these two iso-alleles, the heterozygote for $+^{c}$ being more extreme than that for $+^{2}$. Hence, at low temperature all three heterozygotes differ.

The strikingly large number of flies with *ci* venation in the heterozygotes $+^{3}/ci$ at 14° invites a comparison with homozygous *ci*/*ci*. Such a comparison with various, independent, but isogenic experiments reported earlier[2] demonstrates that *ci* homozygotes are more extreme than the heterozygotes. In the latter the largest class of both females and males is Class 0 while in the homozygotes the mode is at Class 1 or 2.

Tests of Heterozygotes with ci^{W} (*Table 5*).—A very clear differentiation of

TABLE 5

HETEROZYGOTES WITH *ci*^{W} 26°C.

	♀♀					♂♂				
	N	0	1	2	3	*N*	0	1	2	3
$+^{c}/ci^{W}$	311	183	546	40	1	260	224	619	28	...
$+^{2}$ *ey*^{2}/*ci*^{W} + *ey*	239	244	763	157	5	118	223	896	179	...
$+^{3}/ci^{W}$	16	22	405	841	268	18	31	487	783	166

all three iso-alleles is accomplished, at 26°, by combining them with the allele *ci*^{W}. The heterozygotes $+^{c}/ci^{W}$ are distributed significantly more toward the normal phenotype than those of the constitution $+^{2}/ci^{W}$. The $+^{3}/ci^{W}$ flies are still more abnormal than the $+^{2}$ heterozygotes.

Possible Further Differences between the Iso-alleles.—In preliminary work with a homozygous $+^{3}$ stock, not isogenic with the stocks used in the experiments reported above, it was noticed, especially at lower temperature, that many individuals showed abnormally broad wings, sometimes with extra venation between the first and second veins. Occasionally a wing had an extra lobe on its anterior margin. These same abnormalities occurred after the stock had been made isogenic for the first, second and third chromosomes with the $+^{c}$ and $+^{2}$ stocks. Moreover, in the F_1 of the "isogenic" cross $+^{3}/+^{3} \times ci/M$-4 none of the $+^{3}/ci$ individuals had the wing abnormalities, while their $+^{3}/M$-4 sibs were affected. This shows that the peculiar wing abnormalities are due to a recessive, fourth chromosome con-

dition located in that section around the *ci* locus which is deficient in the *M*-4 chromosome. It is likely that the wing characteristics depend on the $+^3$ allele itself and constitute another pecularity of this allele distinguishing it from $+^c$ and $+^2$. Besides the wing abnormality $+^3/M$-4 hemizygotes have enlarged sexcombs, a phenotype not found in the hemizygotes for the two other iso-alleles but which is one of the effects of the mutant allele ci^w. The sexcomb abnormality seems to differentiate further $+^3$ from $+^c$ and $+^2$.

Discussion.—The recognition of three distinct iso-alleles was accomplished in two ways: By testing them (1) in different environments, namely high and low temperatures, and (2) either as hemizygotes, or in heterozygous combinations with mutant alleles. A third kind of test, varying the genetic background, was not used, but might be expected to serve as another tool for the separation of iso-alleles.

To be accurate, the experiments reported do not establish beyond doubt allelic differences at the *ci* locus. They only narrow down the location of the genic differences demonstrated, to the *ci* locus and its immediate surroundings as delimited by the extent of the *M*-4 deficiency. If a locus, or loci, outside the *ci* locus were responsible for the phenotypic differences observed, they would have to be dominant since the tests with heterozygotes rule out recessivity. Thus, an assumption of dominant modifiers of *ci*, located very near to the *ci* locus, is an alternative to regarding the *ci* locus itself as the cause of the differences. At present no means exist to prove this responsibility of the *ci* locus itself, but it seems the most likely assumption to make.

The discovery of iso-alleles is not new. Facts similar to those described here are known for the *vg* and *ct* loci in *D. melanogaster* (Mohr, 1932,[4] Goldschmidt, 1937[5]). Another well-known example is that of two wild-type alleles of the *w* locus which were first recognized by their different somatic mutation rates after x-radiation (Timofeeff-Ressovsky, 1932[6]) and were then shown to give phenotypic differences in triploids in which they were tested with two doses of the mutant white allele (Muller, 1935[7]). The most striking case of numerous iso-alleles so far described is that concerning the bobbed locus in populations of *Drosophila hydei* (Spencer, 1938[8]). Other examples are the "specific modifiers," defined as genes which produce recognizable effects only in the presence of some main gene; they are but iso-alleles of their non-modifying alleles. In this group belong also alleles in some individuals of Crepis (Hollingshead, 1930[9]), Godetia (Hiorth, 1942[10]) and other organisms which act as lethals in species or racial crosses, while another allele of the locus concerned does not have lethal action. Within their own species or race, no differences have been observed between individuals homozygous for either the (potentially) lethal or non-lethal iso-allele. Still other types of iso-alleles are the multiple sex-alleles of Ly-

mantria (Goldschmidt, 1934[11]) and Habrobracon (Whiting, 1943[12]). The peculiarity of the case described in the present paper is not so much the *existence* of the allelic differences but the fact that all three wild-type chromosomes tested were different. This seems to indicate, as did Spencer's findings, that the phenomenon of wild-type iso-allelism may be very frequent. It is probable that iso-allelism responsible for mutant phenotypes likewise is to be encountered. The "gene" then seems variable in many more ways than those responsible for striking effects.

It is possible to analyze somewhat further the differences between the wild iso-alleles of *ci* if the concepts of primary genic action, elaborated earlier,[2, 13] are applied. According to these the action of an allele at the *ci* locus, in regard to wing development, depends (1) on the degree of ability, c, of the allele to interact with a cellular substrate, S, and (2) on an "efficiency factor," e, which measures the effectiveness of interaction in forming a product, P, which functions in the elaboration of the normal phenotype. On the basis of experimental facts, it is postulated that the action of different alleles depends on differences in c and e. As long as S sufficiently exceeds the combining power, c, of an allele, P is considered proportional to the product $c \cdot e$ of any allele.

(*a*) Since at 14° the hemizygotes of all three alleles $+^c$, $+^2$ and $+^3$ are less normal than the homozygotes, i.e.,

$$P(+^c, \text{ or } +^2, \text{ or } +^3/M\text{-}4) < P(+^c/+^c, \text{ or } +^2/+^2, \text{ or } +^3/+^3),$$

it follows that S exceeds the combining power of each allele,

$$c_{+c}, \text{ or } c_{+2}, \text{ or } c_{+3} < S. \tag{1}$$

Since, however, it is known[13] that the sum of the combining powers of $+^3$ and *ci* is greater than S, and that $c_{+3} > c_{ci}$, it follows, that in the homozygote $+^3/+^3$

$$2c_{+3} > S. \tag{2}$$

Hence, the abnormal phenotype at 14° of some $+^3/+^3$ individuals is not due to lack of interaction with the substrate, but to its inefficient utilization.

(*b*) Since the homozygote of $+^3$ is less normal than that for $+^c$ and $+^2$ it follows that

$$c_{+3} \cdot e_{+3} < c_{+c} \cdot e_{+c} \text{ or } c_{+3} \cdot e_{+3}. \tag{3}$$

Since the hemizygotes for $+^2$ and $+^3$, at 14°, are less normal than the $+^c$ hemizygote, i.e.,

$$P(+^2, \text{ or } +^3/M\text{-}4) < P(+^c/M\text{-}4),$$

it follows that

$$c_{+3} \cdot e_{+3} \text{ or } c_{+3} \cdot e_{+3} < c_{+c} \cdot e_{+c}. \tag{4}$$

Jointly, it follows from (3) and (4), that

$$c_{+^1} \cdot e_{+^1} < c_{+^2} \cdot e_{+^2} < c_{+^3} \cdot e_{+^3}. \qquad (5)$$

(*c*) Finally, at 14°, the heterozygote $+^2/ci$ is more normal than the hemizygote $+^2/M$-4 whereas conversely the heterozygote $+^3/ci$ is less normal than the hemizygote $+^3/M$-4. Since it is known that $e_{ci} < e_{+^2}$ or e_{+^3} the facts cited indicate that the "addition" of *ci* to $+^2$ does not result in sufficient competition for *S* to lead to a decrease of *P* as compared with $+^2/M$-4, while the "addition" of *ci* to $+^3$ deprives the latter of so much *S*, and *ci* interacts with *S* with so low an efficiency, that the total amount of *P* is less in the heterozygote $+^3/ci$ than in $+^3/M$-4. This means that

$$c_{+^2} < c_{+^3} \qquad (6)$$

and, in view of (5), that

$$e_{+^2} > e_{+^3}. \qquad (7)$$

No data are available to place the third wild iso-allele, $+^c$, in its proper order according to its *c* and *e* attributes.[14]

The general conclusion is that the wild iso-alleles may vary from each other in both their combining powers and efficiencies as had been shown for mutant alleles.[13] These facts have wider significance! If two iso-alleles, *x* and *y*, differ in their *c* and *e* properties, it is to be expected that the difference in the amounts of *S* utilized by the two alleles (c_x vs. c_y), and the differences in the result of the utilization (e_x vs. e_y) would involve changes in other intracellular reactions. Thus, new points of attack for selective forces would originate. In general, selection should tend to bring the amount of substrate available to an allele to a safe minimum relative to its combining and efficiency powers. The amount of this substrate itself will depend on other genes, some of which may contribute to its elaboration and others which may be involved in its utilization. Any selection for amounts of substrate best fitted to various iso-alleles in one locus will result in the selection of specific iso-alleles of other loci. Hence, there will arise many different genotypes, phenotypically alike, which are due to different systems of iso-alleles. It can hardly be estimated how much of such concealed evolution, due to the kind of "germinal selection" described, is taking place at any time.

Summary.—1. Three stocks, isogenic except for the fourth chromosome, which came from three different sources, are all wild type at 25°–26°. If the three stocks are tested at low temperature, as hemizygotes, or in heterozygous combination with the alleles *ci* and ci^w of the *cubitus interruptus* locus, they are recognized as each carrying a distinct wild-type allele of *ci* (unless the alternative assumption is made of dominant modifiers of *ci* located very close to the locus of *ci*).

2. The existence of iso-alleles, defined as alleles indistinguishable except by special tests, is probably a general phenomenon.

3. The wild-type iso-alleles of *ci* are shown to differ from each other in their ability, *c*, to react with a cellular substrate, *S*, and in their efficiency, *e*, in transforming *S* into a product, *P*, which functions in the elaboration of normal venation. The iso-allele $+^2$ has a smaller *c*, but a larger *e* than the allele $+^3$.

4. It may be expected that natural selection leads to many different systems of iso-alleles in various loci.

[1] Supported in part by a grant from the Rockefeller Foundation.

[2] Stern, C., *Genetics*, **28**, 441–475 (1943).

[3] The chromosome carrying *ci* also contained ey^R. Similarly the chromosome carrying $+^2$ contained ey^2. The ey^R allele is without influence on the expression of *ci*. Except in the tables, references to the *ey* alleles will be omitted.

[4] Mohr, O. L., *Proc. 6th Int. Congr. Genet.* (*Ithaca*), **1**, 190–212 (1932).

[5] Goldschmidt, R., *Univ. Calif. Pub. Zool.*, **41**, 285–296 (1937).

[6] Timofeeff-Ressovsky, N. W., *Biol. Zbl.*, **52**, 468–476 (1932).

[7] Muller, H. J., *Jour. Genetics*, **30**, 407–414 (1935).

[8] Spencer, W. P., *Genetics*, **23**, 170 (1938).

[9] Hollingshead, L., *Ibid.*, **15**, 114–140 (1930).

[10] Hiorth, G., *Zeit. Vererbgsl.*, **80**, 289–349 (1942).

[11] Goldschmidt, R., *Bibliogr. Genetica*, **11**, 1–185 (1934).

[12] Whiting, P. W., *Genetics*, **28**, 365–382 (1943).

[13] Stern, C., and Schaeffer, E. W., *Proc. Nat. Acad. Sci.*, **29**, 351–361 (1943).

[14] It might appear as if the data of tables 3 and 4 relative to $+^C/M$-4 and $+^C/ci$, at 14°, could be analyzed similarly to those relating to $+^2$ and $+^3$. However, as discussed earlier[2] the possibility exists that due to secondary difficulties the observed frequency of *M*-4 types with *ci* venation may be too low as compared to that of non-*M*-4 types so that conclusions should not be drawn from a comparison of the two similar phenotypes. In case of $+^2$ where the observed frequency of abnormal venation is higher in hemizygotes than in heterozygotes any error attributable to misclassification would only tend to decrease but not to simulate an actual difference, and in $+^3$ the differences in distribution are so large as to be beyond doubt.

CHROMOSOME COMPLEMENTS OF SOME SOUTH-BRAZILIAN SPECIES OF DROSOPHILIA

BY TH. DOBZHANSKY* AND C. PAVAN

COLUMBIA UNIVERSITY, NEW YORK, AND UNIVERSITY OF SÃO PAULO, BRAZIL

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For the last several months the writers have been engaged in making a preliminary survey of species of Drosophila which inhabit the states of São Paulo and Rio de Janeiro in Brazil. Among the rather numerous species examined, at least two proved to possess chromosomal complements of unusual interest. The purpose of the present note is to put on record an account of the chromosomes in these two as well as in some related species.

We have collected at Bertioga (state of São Paulo) and at Jacarepagua (Federal District) some females of a species apparently identical with *Drosophila prosaltans* Duda.[1] This species breeds well in the laboratory, and strains have been established without difficulty. Acetic orcein smear preparations of nerve cells of larval ganglia and of larval salivary glands have been made. The nerve cell metaphases show two pairs of V-shaped and one pair of rod-like chromosomes (Fig. 1, *A–E*). The V-shaped chromosomes appear equal-armed, one pair being perhaps slightly larger than the other. The centromere constrictions are usually well pronounced; in some cells (Fig. 1, *C*, *D*) one arm of one of the V's shows a secondary constriction at about the middle of its length, while the other V may show a secondary constriction in one limb in a submedian position. The rod-like chromosomes are about as long as an arm of the V-shaped ones, and have distinctly subterminal centromeres (Fig. 1, *A*, *B*, *E*). Metaphase plates in female and male larvae are indistinguishable; this indicates that the *X*- and *Y*-chromosomes are of about the same size and shape. The salivary gland cells show five long chromosome strands radiating from a well-developed chromocenter. In male larvae two of the five strands are paler than the remaining three. The paler strands represent the *X*-chromosome; the *Y*-chromosome is evidently heterochromatic, and the *X*-strands have no paring mates in male cells.

Cells in which the chromocenter has been crushed by the pressure of the cover slip have been examined in order to determine the correspondence between the salivary and the metaphase chromosomes. In such cells the strands corresponding to a V-shaped chromosome frequently remain connected through the portion of the chromocenter formed by their heterochromatic regions. If a sufficient number of cells are examined this "vital artefact" gives data that are conclusive. In *D. prosaltans* most of the chromocentral mass goes with the two strands denoted in

figure 2 as A and B. These two strands are alike in female and male larvae; therefore, they represent a V-shaped autosome. In some larvae of the Bertioga cultures the B-strand showed two inversions separated by an uninverted section (see Fig. 2). Two other strands (C and X^2 in Fig. 2) remain connected through a small fragment of the chromocenter. Very surprisingly, one of these strands (X^2) proved to be much paler in male than in female larvae, while the other strand (C) is similar in either sex. This difference has been seen in many cells, and it is frequently quite

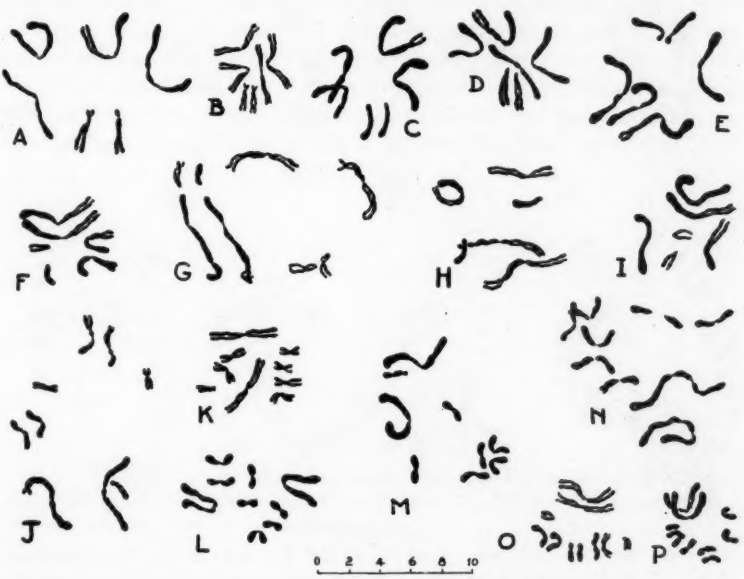

FIGURE 1

Nerve cell metaphases in *Drosophila prosaltans* (*A–E*), *D. sturtevanti* (*F–I*) and *D. annulimana* (*J–P*). The scale below represents 10 micra.

striking. One of the V-shaped metaphase chromosomes consists, therefore, of an autosomal and an X-chromosome limb. The fifth strand (X^1 in Fig. 2) is also paler in male than in female larvae; in cells with a fragmented chromocenter the X^1 strand lies frequently isolated from other chromosomes, the base having a small amount of heterochromatin. The X^1 strand corresponds to the rod-like chromosome of metaphase plates (Fig. 3).

We are forced to conclude that in *D. prosaltans* there are two X- and two Y-chromosomes: one rod-like and free (X^1–Y^1) and the other attached to an autosomal rod, forming with the latter a V-shaped complex (AX^2–

AY^2). The formula of the female is, consequently, $X^1X^1AX^2AX^2$, and of the male $X^1Y^1AX^2AY^2$. Such a chromosomal structure (Fig. 3) is unique in Drosophila; it must lead to a number of genetic complications. If, during the spermatogenesis, the X^1–Y^1 and the AX^2–AY^2 bivalents disjoin independently, four classes of spermatozoa, X^1AX^2, Y^1AY^2, X^1AY^2, Y^1AX^2, must be formed. The two last classes would give rise to inviable zygotes.

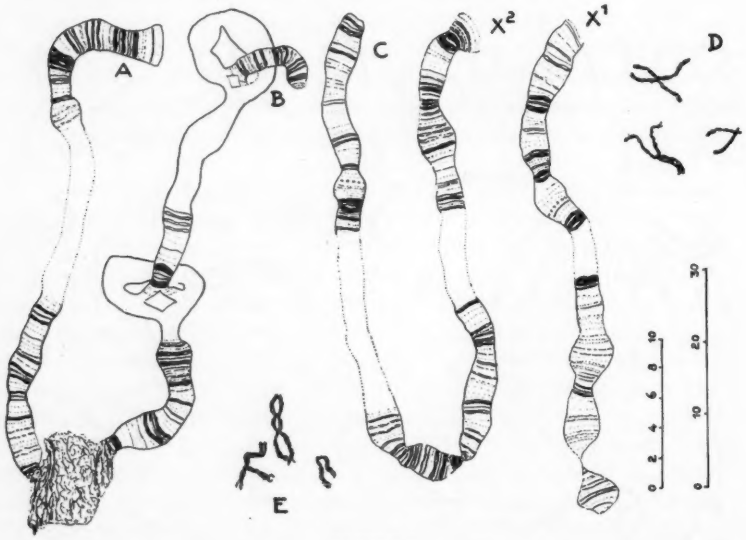

FIGURE 2

Salivary gland chromosomes (*A*, *B*, *C*, X^1 and X^2) and diakinesis configurations (*D* and *E*) in *Drosophila prosaltans*. The disc patterns are represented only in the basal and the terminal portions of the salivary gland chromosomes; the relative lengths of these chromosomes are not reflected in the drawings. The 30 micra scale applies to figures *A*, *B*, *C*, X^1 and X^2, the 10 micra scale to *D* and *E*.

A somewhat analogous situation is observed in *D. miranda*, where the male is X^1YX^2, but where only two types of sperms are produced; the X^1 and X^2 always go to the same pole, and the *Y*-chromosome goes to the opposite pole.[2] The *Y*-chromosome of *D. miranda* contains, however, some material homologous to that contained in the X^1 as well as to that in X^2.[3] How many classes of spermatozoa are formed in *D. prosaltans* is unknown. However, a species producing 50% of inviable eggs could hardly survive in nature; it is extremely likely that X^1AY^2 and Y^1AX^2 spermatozoa are either not formed or are not functional. Unfortunately, *D. prosaltans*, like most other species of Drosophila having highly spiralized testes, is

unfavorable for examination of spermatogenesis. A few smear preparations of the testes of young males have been made, but in none of them have good meta- and anaphases of the first meiotic division been encountered. Some cells showed stages of early diakinesis; three separate bivalents may be distinguished (Fig. 2, *D*, *E*). One of the bivalents consists of two rod-like chromosomes, invariably associated at one end only. The bivalents formed by the V-shaped chromosomes show a variety of structures, in some cases suggesting chiasmata, although this may be only a false appearance. The problem of chromosome disjunction in *D. prosaltans* is, evidently, an open one. Another open problem concerns the behavior of the genes carried in the autosomal pair associated with the X^2–Y^2 chromosomes. The genes in this autosome should show all gradations between the normal autosomal inheritance and inheritance of sex-linked genes having alleles in the *Y*-chromosome (such as bobbed in *D.*

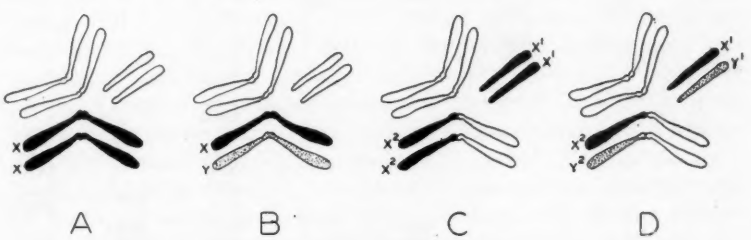

FIGURE 3

A scheme of the chromosome structure in *Drosophila sturtevanti* females (*A*) and males (*B*), and *D. prosaltans* females (*C*) and males (*D*). White—autosomes; black—*X*-chromosomes; stippled—*Y*-chromosomes.

melanogaster). The evolutionary fate of the genes located close to the centromere in the autosome associated with the *Y*-chromosome constitutes a still other interesting problem. Finally, a fourth problem which suggests itself is the origin of the unique heterochromosome mechanism of *D. prosaltans*. Fortunately, this problem can be somewhat clarified by comparison with a related species, namely, *D. sturtevanti* Duda.

We have laboratory strains of *D. sturtevanti*[4] derived from females collected at Bertioga (state of São Paulo) and at Rio de Janeiro (the latter collected by Professor Hugo Souza Lopes). As in *D. prosaltans*, the nerve cell metaphases of *D. sturtevanti* show two pairs of V-shaped and a pair of rod-like chromosomes (Fig. 1, *F–I*). A close examination discloses, however, differences between these two species. In *D. sturtevanti* one of the V-shaped pairs is clearly longer than the other; in favorable cells it can be seen that one of the arms of the smaller V is appreciably longer

than the other. The rod-like chromosome is much shorter than either limb of at least the larger V; in contrast to *D. prosaltans*, the centromere constriction in the rod is not clearly subterminal. Very fortunately, the disc patterns in salivary gland chromosomes of *D. sturtevanti* proved to be sufficiently similar to those of *D. prosaltans*, so that the identification of

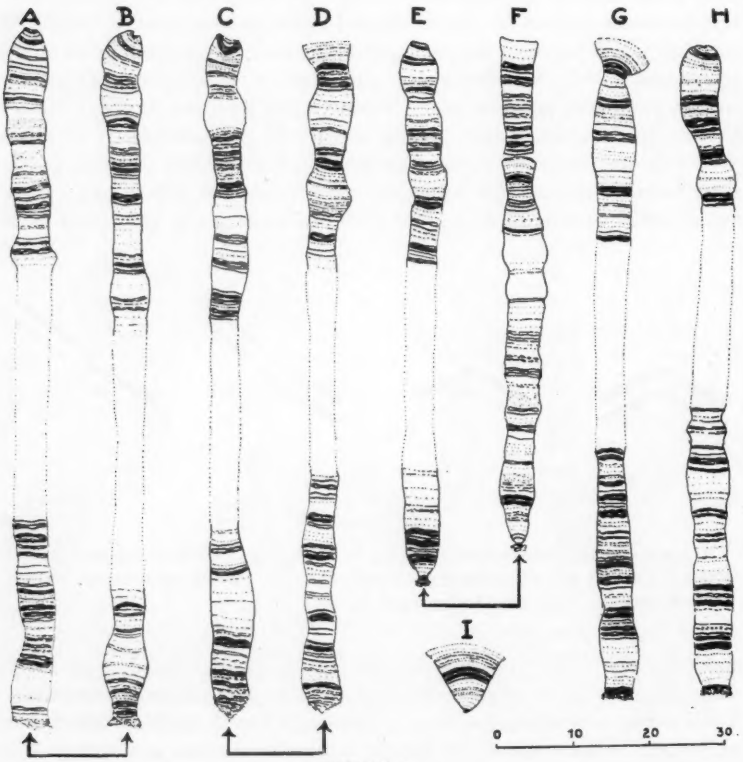

FIGURE 4

Salivary gland chromosomes of *Drosophila annulimana*. Except in the *F*- and *I*-chromosomes the disc patterns are shown only in the basal and the terminal portions; the relative lengths of the chromosomes are not reflected in the drawings. The scale below represents 30 micra.

the corresponding strands can be made without difficulty. In cells with a crushed chromocenter it can be seen that the *A*- and *B*-strands of *D. sturtevanti* are associated through a large chromocentral mass, forming, consequently, a V-shaped complex, like they do in *D. prosaltans*. In contrast to *D. prosaltans*, the *C*-strand of *D. sturtevanti* is frequently isolated

from the other strands, while the X^1 and X^2 strands are connected with each other through a small heterochromatic segment. As in *D. prosaltans*, the X^1 and X^2 strands in male cells of *D. sturtevanti* are paler than the other strands.

It is evident, therefore, that one of the V-shaped chromosomes of *D. sturtevanti* is an *X*-chromosome (or, in the male, an *Y*-chromosome), while the other V and the rod are autosomes (Fig. 3). The type of chromosome complement found in *D. sturtevanti* is "orthodox," and is, virtually certainly, ancestral to the aberrant type of *D. prosaltans*. The origin of the latter form from the former can be visualized only as taking place through reciprocal translocations between the *X*- and *Y*- chromosomes on one hand and an autosomal pair on the other (Fig. 3). V-shaped *X*- and *Y*-chromosomes exchange major portions of one of their limbs for most of a rod-like autosome (*sturtevanti*), giving rise to rod-like *X*- and *Y*-chromosomes and to a V-shaped pair consisting of autosomal and heterochromosomal limbs (*prosaltans*). This change must have involved some situations which, theoretically, might be discriminated against in natural populations. Indeed, the exchange of segments between the autosome and the *X*- and *Y*-chromosomes must have occurred in a species with *sturtevanti*-like chromosomes. At least for a time, translocation heterozygotes of a rather complex kind must have existed, for example, individuals with one V-shaped *X*, one rod-like *X*, one rod-like autosome and one V-shaped autosome-*X*-chromosome complex (and similar heterozygotes for the *Y*-chromosome-autosome translocation). With the disjunction mechanism now present in *D. prosaltans* as yet imperfect, the fertility of such translocation heterozygotes should have been below normal.

It is well known that, in Drosophila, individuals of a race, races of a species and species of the genus frequently differ in inversions of chromosome segments. A great majority of the inversions are paracentric (involving breaks on one side of a centromere), rather than pericentric (involving breaks on two sides of a centromere). The predominance of paracentric inversions in the phylogeny is understandable; paracentric inversion heterozygotes produce few, and pericentric inversion heterozygotes produce relatively many aneuploid gametes. Yet, it can be demonstrated that evolutionary changes sometimes take place through formation of pericentric inversions.[5] In this respect, the chromosome complement of *D. annulimana* Duda is of interest. We possess some cultures of this very large species derived from females collected at Bombas, near Iporanga, state of São Paulo. The nerve cell metaphases in the females show (Fig. 1, *J–P*) ten chromosomes, including a pair of large V's, three pairs of much smaller V's and a pair of Y-shaped or rod-like bodies. The centromere constrictions are so strong that in some cells the two arms of

a V appear as two separate chromosomes; the long V and one of the short V's may show also very strong secondary constrictions in one limb (Fig. 1, *N*). Male cells show nine chromosomes: a pair of large and three pairs of small V's and an unpaired Y or a rod (Fig. 1, *I–M*). *D. annulimana* is, consequently, *XX* in the female and *XO* in the male; this is the second known case of an *XO* species of Drosophila, the first being *D. orbospiracula* Patterson and Wheeler.[6]

The salivary gland cells of *D. annulimana* are very favorable for study. Eight relatively long and one very short chromosome strands can be counted without much difficulty. The disc patterns in the distal and the proximal portions of each strand are shown in Fig. 4, the middle portions being omitted except in shorter strands. The relatively long *A*- and *B*-strands are frequently associated in their proximal parts; they very likely represent the large V-shaped pair of metaphase chromosomes. The *C*-strand is almost always associated with *D*, and *E* with *F*; they probably correspond to two of the smaller V-shaped pairs of the metaphase plates. The *G*-strand is the *X*-chromosomes; *H* and the very small *I* probably belong together, although this has not been established by direct observation.

A majority of species of Drosophila so far cytologically investigated have five long and one very short chromosomal strands in their salivary gland cells, corresponding to the five variously associated or free rods and one dot-like chromosome of metaphase plates. Five free rods and a dot is the most likely ancestral condition of the chromosomal apparatus in the genus Drosophila, and it occurs frequently in species of the repleta group,[6] of which *D. annulimana* is an aberrant member.[7] The minimum of changes which have to be assumed to derive the chromosome complement of *D. annulimana* from the ancestral complement are as follows: (1) junction of two rods to form the large V of *annulimana*; (2) pericentric inversions in two rods with subterminal centromeres; (3) translocation of enough material from one of the rods onto the dot to transform the latter into a small V.

* Visiting Professor at the University of São Paulo, working under the auspices of the Committee for Interamerican Artistic and Intellectual Relations.

[1] Duda (*Arch. f. Naturgesch.*, **91**, 164 (1925)) has described "*Drosophila prosaltans* new species or variety of *saltans* Sturtevant"; the description is based on a single museum specimen from Hohenau, Paraguay. Sturtevant (*Univ. Texas Publ.*, **4213**, 39 (1942)) gave the name *Drosophila sellata* to a species from Guatemala and Mexico which is very close to *D. prosaltans* Duda. Sturtevant's own comment is: "The chief reason for not applying that name (*prosaltans*) to it (*sellata*) is the distribution, plus the fact that I have not seen Duda's material." Our flies fit the description of *D. prosaltans* and differ only slightly from that of *D. sellata*.

[2] Dobzhansky, Th., *Jour. Genetics*, **34**, 135–151 (1937).

[3] Mac Knight, R. H., *Genetics*, **24**, 180–201 (1939).

[4] Described by Duda (*Arch. f. Naturgesch.*, **91**, 167 (1925)) from a single Museum specimen from Mapiri, Bolivia. A very close, possibly identical, species has been named *Drosophila biopaca* by Sturtevant (*Univ. Texas Publ.*, **4213**, 37 (1942)).

[5] Miller, D. D., *Genetics*, **24**, 699–708 (1939).

[6] Patterson, J. T., and Wheeler, M. R., *Univ. Texas Publ.*, **4213**, 67–109 (1942).

[7] *Drosophila annulimana* is described by Duda (*Arch. f. Naturgesch.*, **91**, 117 (1925)) as a member of his subgenus Paradrosophila. As delimited by Duda, this subgenus is not a natural group; it includes most diverse forms having in common only a single character, namely, prescutellar bristles. *Paradrosophila annulimana* is much closer to species of the repleta group of the subgenus Drosophila than to such representatives of Paradrosophila as *bromeliae* Sturtevant. A new species close to *bromeliae* is found in São Paulo. It has three pairs of about equally long V-shaped chromosomes.

A TYPE OF UNIVERSAL ARITHMETICAL FORMS

BY E. T. BELL

DEPARTMENT OF MATHEMATICS, CALIFORNIA INSTITUTE OF TECHNOLOGY

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1. By a few obvious changes in the wording, all that follows may be restated for any unique factorization domain. The type of forms considered is defined in §§2, 3.

If the integer $n \neq 0$ is expressible in the form $x^a y^b \ldots w^c$, where all the letters denote integers, $a, b, \ldots, c$ are constants and (without loss of generality) are all different, we shall say that n has the *index* $[a, b, \ldots, c]$, and write

$$I(n) = [a, b, \ldots, c].$$

The order of $a, b, \ldots, c$ within the bracket is immaterial.

The set of all integers having the index $[a, b, \ldots, c]$ will be written $I^{-1}[a, b, \ldots, c]$. Hence if at least one of $a, b, \ldots, c$ is 1, $I^{-1}[a, b, \ldots, c]$ is $I^{-1}[1]$, the set of all integers except zero.

Let $p_1, \ldots, p_s$ be the distinct positive prime divisors of $n > 1$, and let

$$t = \prod_{i=1}^{s} p_i^{t_i}, \qquad t = x^a y^b \ldots w^c,$$

be the prime decomposition of t and a representation of t with index $[a, b, \ldots, c]$ and $x, y, \ldots, w > 0$. Denote by $L(t;\ a, b, \ldots, c)$ the number of such representations. Then, on replacing $x, y, \ldots, w$ by their prime decompositions, it is evident that

$$L(t;\ a, b, \ldots, c) = \prod_{i=1}^{s} (t_i;\ a, b, \ldots, c),$$

in which $(t_i;\ a, b, \ldots, c)$ is the number of sets of solutions $(x_i, y_i, \ldots, w_i)$ of

$$ax_i + by_i + \ldots + cw_i = t_i$$

in integers ≥ 0. Thus $L(t;\ a, b, \ldots, c)$ is defined for integers $n > 1$.

By definition, $L(1;\ a, b, \ldots, c)$ is the number of solutions of $1 = x^a y^b \ldots w^c$ in positive integers; $L(1;\ a, b, \ldots, c) = 1$.

If $t < 1$, $L(t;\ a, b, \ldots, c)$ is defined as the number of representations of t in the form $x^a y^b \ldots w^c$ by integers $x, y, \ldots, w$; $L(t;\ 2a, 2b, \ldots, 2c) = 0$. If $t < 1$ and $a, b, \ldots, c$ are not all even, let precisely g of them be odd. Then by changes of an odd number of signs of the variables having these g odd exponents,

$$L(t;\ a, b, \ldots, c) = g_1{}^2 L(-t;\ a, b, \ldots, c), \qquad t < 1,$$

where g_1 is the greatest integer $\leq (g + 1)/2$.

If the number of variables $x, y, \ldots, w$ is h, it follows that

$$L(t;\ a, b, \ldots, c) = O((t_1 \ldots t_s)^{h-1}), \qquad |t| > 1.$$

2. A *form* $F(x, y, \ldots, w)$ in the variables $x, y, \ldots, w$ is defined here to be a polynomial in the variables with integer coefficients and no constant term. (In the case of any unique factorization domain the coefficients are in the domain.) The number of terms in a form will be called its *extent*.

If integer values $x', y', \ldots, w'$, *all different from zero*, of $x, y, \ldots, w$ can be found such that $n = F(x', y', \ldots, w') \neq 0$, we shall say that n is *represented* by $(x', y', \ldots, w')$ in $F(x, y, \ldots, w)$. It is to be noted that representations of zero are undefined. (If zero values of the variables were admitted, the problem of representation for the type of forms considered here would reduce to the case discussed.)

If all integers in the set $I^{-1}[a, b, \ldots, c]$ are represented in $F(x, y, \ldots, w)$, we say that the form is *universal for the set*, write

$$F(x, y, \ldots, w), \qquad [a, b, \ldots, c],$$

and call $[a, b, \ldots, c]$ an *index* of $F(x, y, \ldots, w)$. In particular,

$$F(x, y, \ldots, w), \qquad [1, b, \ldots, c]$$

states that all integers $\neq 0$ are represented as defined in $F(x, y, \ldots, w)$.

For all the forms considered a lower limit for the number $L(t)$ of representations of each integer t in the set $I^{-1}[a, b, \ldots, c]$ for which $F(x, y, \ldots, w)$ is universal is $L(t) \equiv L(t;\ a, b, \ldots, c)$. In particular, if all integers $t \neq 0$ are represented, t has at least $L(t;\ 1, b, \ldots, c)$ representations.

3. The general form of extent s in the variables $x_1, x_2, \ldots, x_n$ of the type considered here may be written

$$\sum_{i=1}^{s} d_i x_1^{a_i} x_2^{b_i} \ldots x_n^{c_i},$$

where the d_i are integers $\neq 0$ and the exponents are integers subject to the conditions

$$a_i, b_i, \ldots, c_i \geq 0, \qquad i = 1, \ldots, s;$$

$$\prod_{i=1}^{s} a_i = \prod_{i=1}^{s} b_i = \ldots = \prod_{i=1}^{s} c_i = 0;$$

$$\sum_{i=1}^{s} a_i > 0, \qquad \sum_{i=1}^{s} b_i > 0, \ldots, \qquad \sum_{i=1}^{s} c_i > 0.$$

4. Before indicating the method of proof we give some examples for forms of extent 3. Similar results for forms of any odd extent are readily obtainable by the method indicated; forms of even extent require other considerations, which apply also to forms of odd extent.

For a form representing all integers $t \neq 0$ an index is given so that the limit $L(t)$ may be written down. Those of these forms in which two signs are positive and one negative represent any $t > 0$ by at least $L(t)$ sets of values all > 0 of the variables. Any integer values which make the exponents ≥ 0 may be assigned to m, n; any zeros in the resulting index symbol are to be suppressed.

$$x^{m+1}y^n + yz^{m+1}w^{n-1} - w^n z x^m, \qquad [n-1, n, m+1, nm+n+1, nm+m+1]. \quad (1)$$

$$z(x^{m-1}y^{n+1} + w^{n+1}) - yx^m w^n, \qquad [1, m+n, m+2n]. \quad (2)$$

$$xy^n z^{m-1} - z^m w^n + w^{n-1} y x^m, \qquad [1, n, m, nm+m-1]. \quad (3)$$

$$x(y^{n+1} + z^{m+1}) - y^m z^n, \quad (4)$$

of which an index is the set of all the numbers common to $[n+1, n+2, \ldots, 2n+1]$, $[m+1, m+2, \ldots, 2m+1]$.

$$x^m y^n z + z^2 y^{n-1} w^m + w^n x^{m+1}, \qquad [n, m+1, n+m+1]. \quad (5)$$

All of equations (1) to (3), and (5) are homogeneous. Another example of a nonhomogeneous universal with two arbitrary integer exponents is

$$x^n y^{m-1} + y^m z + z^2 x, \qquad [1, 3, 5, \ldots, 2n+1]. \quad (6)$$

The representations in equations (7) and (8) contain integer parameters:

$$x^m y^2 z^{n-1} + z^n y w^m + w^{m+n} x, \qquad [1, m+n, m+n+1], \quad (7)$$

has 1-parameter representations of all integers $t \neq 0$; every t^4 has at least $L(t;\ 1, 2, 3)$ 4-parameter representations in

$$x^4 + y^4 + xyzw. \quad (8)$$

The next four are typical of a class in which the sum of two terms can be written $x(y^n + w^n)$:

$$\begin{aligned} &x(y^3 + w^3) + zyw, && [1, 3, 4, 6]; \\ &x(y^3 + w^3) + zy^2w^2, && [1, 3, 4, 5]; \\ &x(y^5 + w^5) + zyw, && [1, 5, 6, 10, 15, 20]; \\ &x(y^5 + w^5) + zy^4w^4, && [1, 2, 6, 7, 8, 9, 10]. \end{aligned} \tag{9}$$

As examples of forms with index different from [1],

$$\begin{aligned} &xy^3 - yz^3 + zx^3, && [2]; \\ &x^4y + y^2xz^2 + z^5, && [3, 5, 12, 17]; \\ &x^5 + zx^2y^2 + y^5, && [2, 3, 5]; \\ &x^4y^2 + yz^5 + z^3x^3, && [5, 6, 11, 13]. \end{aligned} \tag{10}$$

5. A sufficient indication of a method for obtaining indices of forms of odd extent of any degree in any number of variables is given by two simple remarks: $I^{-1}[1]$ is the set of all integers; a solution of any one of a given set of equations $E_1 = 0, \ldots, E_s = 0$ is a solution of $E_1 \ldots E_s = 0$. (All of the examples in §4 refer to the case $s = 2$.) When at least one of the equations, say, $E_j = 0$, can be written $E_j' = E_j''$, where E_j', E_j'' are monomials in the variables and have no variable in common, the complete integer solution of $E_j = 0$ expresses each of the variables as a monomial in integer parameters. Applying this and the known methods for finding the solutions described to the second remark above, we find the indices. In all of the examples in §4 the coefficients in the forms are ± 1. Any numerical coefficients may be introduced as in extending the solution of $E_j' = E_j''$ to that of $a_jE_j' = b_jE_j''$, where a_j, b_j are any given integers. If each of $E_1 = 0, \ldots, E_s = 1$, $s > 1$ can be written in the same form as E_j, the extent of the form whose index is to be found is $2^s - 1$.

THE APOLLONIAN PACKING OF CIRCLES

BY EDWARD KASNER AND FRED SUPNICK

DEPARTMENT OF MATHEMATICS, COLUMBIA UNIVERSITY

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A theorem of the senior author concerning the covering of the plane by circles is established in *Scripta Mathematica* for March, 1943.[1] In this paper we give an analytic proof of the same theorem. The subject is also of interest in geology (packing of sand, porosity problems) and other fields of science.

1. *Definitions and Statement of the Theorem.* Let us effect a covering

of the plane with mutually external, equal circles each tangent to six others. We call this configuration the *circlex*. That part of the plane which is external to the circles of our covering consists of curvilinear triangles. In each of these we inscribe a circle, and continually keep inscribing circles in each curvilinear triangle obtained thereafter *ad infinitum*. We call the latter configuration an *Apollonian packing of circles in the plane* (*or hypercirclex*).

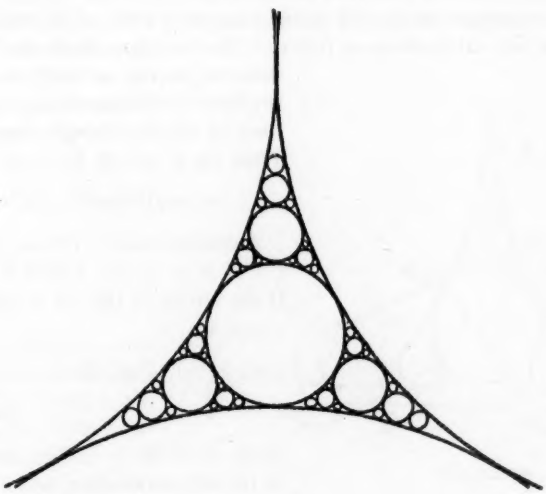

FIGURE 1

Let $T(a, b, c)$ denote the curvilinear triangle formed by the three mutually and externally tangent circles C_a, C_b, C_c with radii a, b, c, respectively. The term *Apollonian packing of circles in* $T(a, b, c)$ is defined similarly, i.e., we inscribe a circle in $T(a, b, c)$ and continually keep inscribing circles in each resulting curvilinear triangle (see Fig. 1).

Let a set G of mutually external circles each lying in $T(a, b, c)$ be given. Then the term *vacancy of* $T(a, b, c)$ *relative to* G signifies the set of points in or on $T(a, b, c)$ which are not interior to an element of G.

We now state the

THEOREM: *The area of the vacancy of an Apollonian packing of circles in any curvilinear triangle is zero. In other words, the sum of the areas of the infinity of circles of an Apollonian packing in* $T(a, b, c)$ *is equal to the area of* $T(a, b, c)$.

We note that since our theorem is true, then because of the symmetrical properties of our initial covering of the plane, it is an immediate conse-

quence that the area of the vacancy of an Apollonian packing of circles in the plane is zero.

Our method of proof is different from that of the previous paper in the sense that we choose a different sequence of diminishing vacancies, it being easier to determine a bound for the ratio of diminution at each stage.

Let H denote the set of circles associated with an Apollonian packing in $T(a, b, c)$.

Let $C(x, y, z)$ denote the circle inscribed in $T(x, y, z)$.

The most natural method of exhausting the circles of H would be to group them into subdivisions as follows: We inscribe a circle in $T(a, b, c)$; then we inscribe a circle in each of the three curvilinear triangles formed; then in the 3^2 triangles formed, etc. Thus, for $n = 1, 2, 3, \ldots, i, \ldots$

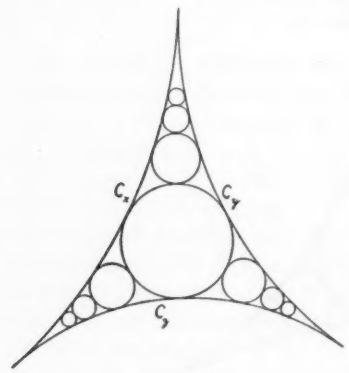

FIGURE 2

1st subdivision: $C(a, b, c)$

2nd subdivision: $C(C(a, b, c), b, c)$, $C(C(a, b, c), a, c)$, $C(C(a, b, c), a, b)$. If the circles of the $(n - 1)$st subdivision are:

$$C(a_1, b_1, c_1),\ C(a_2, b_2, c_2),\ \ldots,\ C(a_k, b_k, c_k)$$

where $k = 3^n - 2$, then the circles of the nth subdivision are:

$$C(C(a_j, b_j, c_j), b_j, c_j),\ C(C(a_j, b_j, c_j), a_j, c_j),\ C(C(a_j, b_j, c_j), a_j, b_j)$$

where $j = 1, 2, \ldots, 3^{n-2}$. But, the most natural procedure is not the most convenient in this case.

We define what we shall call a *necklace packing* (see Fig. 2) of a curvilinear triangle $T(a, b, c)$. It is the set of circles which comprises $C(a, b, c)$, the three circles inscribed in the curvilinear vertex triangles, the three circles inscribed in the new vertex triangles, and so on. Thus for $n = 1, 2, \ldots, i, \ldots$ it contains the circles belonging to all of the following subdivisions:

1st subdivision: $C(a, b, c)$

2nd subdivision: $C(C(a, b, c), b, c)$, $C(C(a, b, c), a, c)$, $C(C(a, b, c), a, b)$.

If the circles of the $(n - 1)$st subdivision are:

$$C(a_1, b, c),\ C(a_2, a, c),\ C(a_3, a, b)$$

then the circles of the nth subdivision are

$$C(C(a_1, b, c), b, c),\ C(C(a_2, a, c), a, c),\ C(C(a_3, a, b), a, b).$$

2. *The Fundamental Lemma.* We now establish the following

LEMMA: *A necklace packing of a curvilinear triangle $T(x, y, z)$ covers more than one-half the area of $T(x, y, z)$.*[2]

Let us effect a necklace packing of $T(x, y, z)$. Let U and V denote any two tangent circles of the necklace set, with radii u, v and centers s_u, s_v, respectively. We connect s_u to s_v with a line segment. We also draw line segments from the points s_u, s_v to the points x_u, x_v at which U and V touch one of the sides of $T(x, y, z)$, say C_z (see Fig. 3). We note that

$$\sphericalangle\, x_u s_u s_v + \sphericalangle x_v s_v s_u \leq \pi. \quad (1)$$

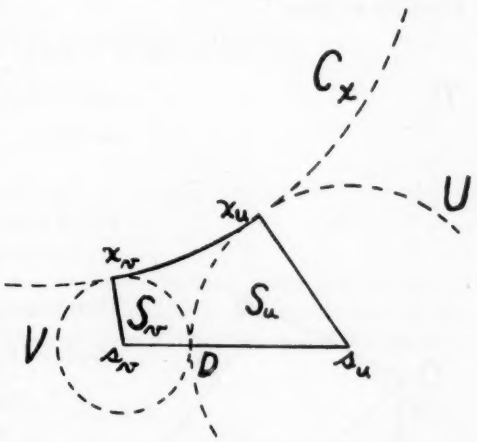

FIGURE 3

Let $K[R]$ denote the area of the region R. We show that $K[T(u, v, x)]$ *is less than the sum of the areas of the sectors S_u, S_v of U and V included in the triangle* (s_u, s_v, s_z), where s_z is the center of C_z. Let $LT(u, v, x)$ denote the linear triangle joining the vertices of the curvilinear triangle $T(u, v, x)$. Then

$$K[T(u, v, x)] < K[LT(u, v, x)]. \quad (2)$$

Now $LT(u, v, x)$ divides triangle (s_u, s_v, s_z) into four mutually exclusive triangles. We show that $K[LT(u, v, x)]$ *is less than the sum of the areas of those two of the other three triangles which contain the points s_u and s_v respectively.*

Let us consider the set of triangles with vertices R, S and T, where the points R and S are fixed, the segment $\overline{RS}$ being of constant length k, and where T may be any point in one of the half planes determined by the line going through R and S (see Fig. 4). We also admit the case where $\sphericalangle RTS$ is zero, or $RT \| TS$. Let P be any point on $\overline{RS}$ not an end-point. Let the segment $\overline{RP} = \alpha$ and the segment $\overline{PS} = \beta$. We lay off a segment $\overline{RQ}$ equal to α or $\overline{RT}$ and a segment $\overline{SW}$ equal to β on $\overline{ST}$. We show that

the area δ of ΔPQW is less than or equal to the sum μ of the areas of ΔRPQ and ΔPWS.[3] Now

$$\delta = 2\alpha\beta \sin (R/2) \sin (S/2) \sin ((R + S)/2) \tag{3}$$

and

$$\mu = (\alpha^2/2) \sin R + (\beta^2/2) \sin S. \tag{4}$$

We must show that $\delta \leq \mu$ for

$$\sphericalangle R + \sphericalangle S \leq \pi. \tag{5}$$

From (5) we have

$$\sin (S/2) \leq \sin((\pi - R)/2) = \cos (R/2) \tag{6}$$

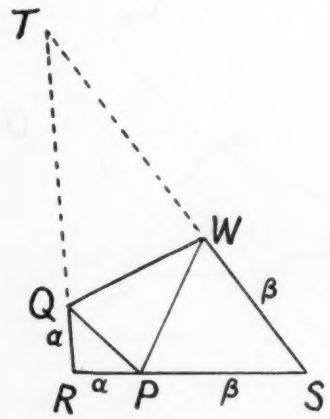

FIGURE 4

and

$$\sin (R/2) \leq \sin ((\pi - S)/2) = \cos (S/2). \tag{7}$$

Let us first consider the case where

$$\sin R \leq \sin S. \tag{8}$$

Then from (6) and (8) we have

$$\begin{aligned} &2\alpha\beta \sin (R/2) \sin (S/2) \sin ((R + S)/2) \\ &\leq 2\alpha\beta \sin (R/2) \sin (S/2) \\ &\leq 2\alpha\beta \sin (R/2) \cos (R/2) \\ &= \alpha\beta \sin R \\ &\leq ((\alpha^2 + \beta^2)/2) \sin R \\ &= (\alpha^2/2) \sin R + (\beta^2/2) \sin R \\ &\leq (\alpha^2/2) \sin R + (\beta^2/2) \sin S. \end{aligned} \tag{9}$$

Now let us consider the case where

$$\sin S < \sin R. \tag{10}$$

This case may be established by a sequence of inequalities as in (9), if we now use (7) where we have used (6), and (10) where we have used (8).

Now let D be the point at which the circles U and V are tangent. Then as a consequence of the latter argument we have

$$\begin{aligned} K[T(u, v, x)] < K[LT(u, v, x)] &\leq K[\Delta(s_u, D, x_u)] + K[\Delta(s_v, D, x_v)] \\ &< K[S_u] + K[S_v]. \end{aligned} \tag{11}$$

Now let $T(x, y, z)$ be completely subdivided as above. Thus, we join the centers of any two tangent circles of the necklace set associated with $T(x, y, z)$ with linear segments. And, if W is any circle of this necklace

set, then we join its center to the points where W contacts C_x, C_y or C_z, with linear segments. Thus, $T(x, y, z)$ is the sum of a denumerable set of non-overlapping regions $\{A_i\}$, each bounded by three linear segments and a subarc of C_x, C_y or C_z (see solid line in Fig. 3). Let S_{i1} and S_{i2} be the two circular sectors included in A_i, and t_i the remaining curvilinear triangle. To each A_i we may now apply (11). Then

$$\begin{aligned} K[T(x, y, z)] &= \sum_{i=1}^{\infty} K[A_i] \\ &= \sum_{i=2}^{\infty} (K[S_{i1}] + K[S_{i2}] + K[t_i]) \\ &= \sum_{i=1}^{\infty} (K[S_{i1}] + K[S_{i2}]) + \sum_{i=1}^{\infty} K[t_i] \\ &\geq 2 \sum_{i=1}^{\infty} K[t_i]. \end{aligned} \tag{12}$$

Therefore,

$$(\sum_{i=1}^{\infty} K[t_i])/K[T(x, y, z)] \leq 1/2. \tag{13}$$

This proves our fundamental lemma.

3. *Proof of the Theorem.* Let us effect a necklace packing of our curvilinear triangle $T(a, b, c)$. Let $\omega = K[T(a, b, c)]$. Denote the sum of the areas of the circles of this necklace set by σ_1. Then the area of the vacancy v_1 is

$$\omega - \sigma_1 \leq \omega/2. \tag{14}$$

But v_1 consists of a denumerable set of curvilinear triangles. In each of these effect a necklace packing and let σ_2 denote the sum of the areas of all these necklace sets. Then the area of our second vacancy v_2 is

$$\omega - (\sigma_1 + \sigma_2) \leq \omega/2^2. \tag{15}$$

Proceeding similarly, the area of the nth vacancy v_n is

$$\omega - \sum_{i=1}^{n} \sigma_i \leq \omega/2^n \tag{16}$$

and

$$\lim_{n \to \infty} \omega - \sum_{i=1}^{n} \sigma_i = 0. \tag{17}$$

But $\sum_{i=1}^{\infty} \sigma_i$ is the sum of the areas of the Apollonian packing of $T(a, b, c)$. Thus the area of the vacancy of the Apollonian packing of $T(a, b, c)$ is zero; or, the sum of the areas of the circles of an Apollonian packing in $T(a, b, c)$ is equal to the area of $T(a, b, c)$. Or, if we borrow the geological

term *porosity*, and let it designate the ratio of the area of the vacancy (relative to a set of mutually external circles in $T(a, b, c)$) to the area of $T(a, b, c)$, then we may assert that the porosity of an Apollonian packing of circles in $T(a, b, c)$ is zero.

The packing of spheres requires methods which are essentially different from that of the present two-dimensional discussion. This question will be the subject of another paper.

In the case of the best covering of the plane with equal circles (no matter how small), the covering ratio is 0.9069, and thus the porosity is 0.0931. The covering ratio of space with equal spheres (no matter how small) is 0.7404 and thus the porosity is 0.2596 (normal packing). This is well known in geological literature in connection with the packing of sand and the amount of oil contained in the oil sands. Our work deals with the packing of unequal spheres, and we find that the porosity may be made as small as we please.[4]

[1] Kasner, E., Comenetz, G., and Wilkes, J., "The Covering of the Plane by Circles," *Scripta Mathematica*, **9**, 19–25 (1943).

[2] We have good reason to believe that the ratio of the sum of the areas of the circles of a necklace packing in any $T(x, y, z)$, to the area of $T(x, y, z)$, is greater than $\pi/4 = 0.7854$ and less than 0.8223. This problem will be treated in another paper. We thank Aida Kalish and Hüseyin Demir for assistance in the calculation of the upper limit 0.8223.

[3] We note that this part of the proof is somewhat more general than that actually required by the lemma.

[4] Kasner, E., "Note on Non-Apollonian Packing in Space," *Scripta Mathematica.*, **9**, 26 (1943). See also *Science*, Oct. 16, 1942. Incidentally we note the following new theorem: In any circular triangle (bounded by mutually tangent circles) the conformal bisectors of the three horn angles (these are necessarily circles) are concurrent. The resulting point we call the *inversive center* of the triangle. For the bisection of analytic horn angles see the senior author's papers an conformal geometry.

CONCERNING CONTINUA WHICH HAVE DENDRATOMIC SUBSETS

BY R. L. MOORE

DEPARTMENT OF PURE MATHEMATICS, UNIVERSITY OF TEXAS

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The subset K of the compact continuum M is said to be a *dendratomic* subset of M if there exists an upper semicontinuous collection G of mutually exclusive continua filling up M such that (1) K is an element of G, (2) G is a dendron with respect to its elements and (3) if H is an upper semicontinuous collection of mutually exclusive continua filling up M such

that H is a dendron with respect to its elements then every element of G is a subset of some element of H.

The compact continuum M will be called a *web* provided there exist two upper semicontinuous collections H_1 and H_2 of mutually exclusive continua such that (1) each of these collections fills up M, (2) each of them is a dendron with respect to its elements and (3) there exists an uncountable subcollection W of the collection H_1 such that no element of W is a subset of any element of H_2. A compact continuum is said to be *webless*[1] if it contains no web.

THEOREM 1. *If the compact continuum M is a web there exist two upper semicontinuous collections Z_1 and Z_2 of mutually exclusive continua such that (1) for each i ($i = 1, 2$), Z_i fills up M and is an arc with respect to its elements, and (2) every continuum of Z_1 intersects every continuum of Z_2.*

Proof. Let H_1, H_2 and W denote collections satisfying, with respect to M, all of the conditions stated in the above definition of a web. There exist two mutually exclusive closed point sets L and T and an uncountable subcollection W_1 of W such that every continuum of the collection W_1 intersects both L and T. There exists a sequence γ of continua of the collection W_1 which converges to a continuum a_1 of that collection. There exist two points A and B belonging to a_1 but not to the same continuum of the collection H_2. Let α_2 and β_2 denote the continua of H_2 that contain A and B, respectively. Since H_2 is a continuous curve with respect to its elements there exists an arc $\alpha_2\beta_2$ of elements of H_2 having α_2 and β_2 as its end-elements. Since H_2 fills up M and is a dendron with respect to its elements, the continua α_2 and β_2 are separated from each other in M by every continuum of the collection $\alpha_2\beta_2$ other than themselves. Let a_2 denote a continuum of the collection $\alpha_2\beta_2$ distinct from α_2 and β_2, let b_2 denote a continuum of this collection that separates a_2 from β_2 in M and let a_2b_2 denote the arc of elements of $a_2\beta_2$ whose end-elements are a_2 and b_2. Let D_A denote the set of all points of M that are separated from b_2 in M by a_2 and let D_B denote the set of all those that are separated from a_2 in M by b_2. The point sets D_A and D_B are open subsets of M containing A and B, respectively. Since γ converges to a_1 there exists a continuum b_1 belonging to γ, distinct from a_1 and intersecting both D_A and D_B. There exists an arc a_1b_1 of elements of H_1 having a_1 and b_1 as its end-elements.

For each i ($i = 1, 2$), let Z_i denote the collection of all continua z such that, for some continuum x of a_ib_i, z is the sum of all continua y of H_i such that y is not separated from x in M by any continuum of the collection a_ib_i.

THEOREM 2. *In order that a compact continuum should have dendratomic subsets it is necessary and sufficient that it should not be a web.*

Proof. If the statement of theorem 35 of page 360 of chapter V of my book, "Foundations of Point Set Theory,"[1] is strengthened by the substitution of "compact continuum which is not a web" in place of "web-

less compact continuum," the resulting theorem may be proved by an argument identical with the one there given to prove theorem 35 except for the substitution of "not a web" for "webless" in its tenth line. With the help of theorem 37 of that chapter it follows that if a compact continuum is not a web then it has dendratomic subsets.

Suppose, now, that the compact web M has dendratomic subsets. Let Z_1 and Z_2 denote collections satisfying, with respect to M, all of the conditions of theorem 1 of the present paper. For each i ($i = 1, 2$), let x_i and y_i denote two continua of the collection Z_i. By hypothesis there exists an upper semicontinuous collection G of mutually exclusive continua filling up M such that (1) G is a dendron with respect to its elements and (2) if a continuum of the collection G intersects a continuum which belongs either to Z_1 or to Z_2 then it lies wholly in that continuum. Let a and b denote continua of the collection G which are subsets of $x_1 \cdot x_2$ and $x_1 \cdot y_2$, respectively. Let U denote the collection of all continua of G that are subsets of x_1 and let V denote the collection of all those that are subsets of the continuum $x_2 + y_1 + y_2$. With respect to the elements of G, U and V are intersecting subcontinua of G whose common part is the set of all elements of G that are subsets either of $x_1 \cdot x_2$ or of $x_1 \cdot y_2$ and clearly this set is not a connected set of elements. But G is a dendron with respect to its elements and no dendron contains two intersecting continua whose common part is not connected. Thus the supposition that M has dendratomic subsets leads to a contradiction.

Definition. The compact continuum M is said to be a *triod*[2] if it is the sum of three continua such that the common part of all three parts of them is both a non-vacuous subcontinuum of each of them and the common part of every two of them.

THEOREM 3. *In order that the compact continuum M should be a triod it is necessary and sufficient that it should contain a continuum K such that $M - K$ has more than two components.*

Proof. This condition is clearly necessary. It is also sufficient. For if K is a subcontinuum of M such that $M - K$ has at least three components then (1) by a theorem of F. B. Jones, $M - K$ is the sum of three mutually separated point sets T_1, T_2 and T_3 and (2) the point sets $K + T_1$, $K + T_2$ and $K + T_3$ are continua and K is the common part of every two of them.

THEOREM 4. *The compact continuum M is a triod if it contains three continua α, β and γ such that* (1) *α and β are mutually exclusive,* (2) *α separates M, and* (3) *γ intersects α and separates two points of β from each other in M.*

Proof. By hypothesis, $M - \gamma$ is the sum of two mutually separated point sets T_1 and T_2 such that $T_1 \cdot \beta$ and $T_2 \cdot \beta$ exist and $M - \alpha$ is the sum of two mutually separated point sets H and K such that H contains β.

The point sets $T_1 \cdot H + \alpha + \gamma$, $T_2 \cdot H + \alpha + \gamma$ and $K + \alpha + \gamma$ are continua, their sum is M and the common part of every two of them is the continuum $\alpha + \gamma$.

It is not true that the compact continuum M is a triod if it contains two continua β and γ such that β separates two points of γ from each other in M and γ separates two points of β from each other in M. If the continuum M is the sum of three arcs AXB, AYB and AZB such that the common part of every two of them is $A + B$ then M contains two such continua β and γ. But it is not a triod.

THEOREM 5. *Every web is a triod.*

Proof. Suppose M is a web. Let Z_1 and Z_2 denote two upper semicontinuous collections satisfying, with respect to M, all of the conditions listed in the statement of theorem 1. Let α and β denote two continua belonging to but not end-elements of, the collection Z_1 and let γ denote one belonging to Z_2 but distinct from its end-elements. The continuum γ intersects both α and β and $M - \gamma$ is the sum of two mutually separated point sets H and K. Let X and Y denote points belonging to H and K, respectively, and let h and k denote the continua of the collection Z_2 that contain X and Y, respectively. The continua h and k are subsets of H and K, respectively. But β intersects both of these continua. Thus $\beta \cdot H$ and $\beta \cdot K$ exist. The continuum γ separates $\beta \cdot H$ from $\beta \cdot K$ in M. Therefore, by theorem 4, M is a triod.

THEOREM 6. *If the compact continuum M is not a triod it has dendratomic subsets and they are arcatomic[8] subsets of M.*

Proof. That M has dendratomic subsets follows directly from theorems 2 and 5. With the aid of the fact that every compact dendron which is not an arc is a triod it follows that if an upper semicontinuous collection of mutually exclusive continua fills up M and is a dendron with respect to its elements then it is an arc with respect to its elements. Hence the dendratomic subsets of M are arcatomic subsets of it.

THEOREM 7. *If A and B are two end-points of the compact dendron M there exists an upper semicontinuous collection H of mutually exclusive continua filling up M such that, with respect to its elements, H is an arc whose end-elements are A and B.*

Proof. The dendron M contains an arc AB. For each point P of AB, let h_P denote P, or the continuum obtained by adding to P every component of $M - AB$ of which P is a limit point, according as there does not or does exist at least one such component. Let H denote the collection of all such continua h_P. This collection is upper semicontinuous, it is an arc with respect to its elements and its end-elements are h_A and h_B. But $h_A = A$ and $h_B = B$.

THEOREM 8. *If a compact continuum M has arcatomic subsets then not only are they dendratomic subsets of M but every upper semicontinuous collec-*

tion of mutually exclusive continua which fills up M and which is a non-degenerate dendron with respect to its elements is also an arc with respect to its elements.

Proof. Let G denote the collection of all arcatomic subsets of M. Suppose H is an upper semicontinuous collection of mutually exclusive continua filling up M such that H is a dendron with respect to its elements. Suppose this dendron has three end-elements, α, β and γ. With the help of Theorem 7 and certain theorems of the above-mentioned book it may be seen that there exists an upper semicontinuous collection Q of mutually exclusive continua filling up M such that (1) every continuum of H is a subset of some continuum of Q, (2) α and β belong to Q, and (3) with respect to its elements Q is an arc whose end-elements are α and β. It follows that each of the continua α, β and γ contains every continuum of the collection G which it intersects. But if a, b and c denote the set of all the continua of G that lie in α, the set of all those that lie in β and the set of all those that lie in γ, respectively, then, *with respect to the elements of G regarded as points*, a, b and c are three mutually exclusive subcontinua of G and $G - a$, $G - b$ and $G - c$ are all connected. This is contrary to the fact that G is an arc with respect to its elements. Hence H has only two end-elements. Therefore it is an arc with respect to its elements. It follows that every element of G is a dendratomic subset of M.

It is not true that if a compact continuum has arcatomic subsets then it is not a triod.

Example. In a Euclidean plane let AB denote a straight line interval and let T denote a totally disconnected perfect subset of AB containing both A and B. Let $Q_1, Q_2, Q_3, \ldots$ denote the components of $AB - T$. For each n let A_n and B_n denote the end-points of the segment Q_n, A_n being between A and B_n. For each point P of $T - A$ let J_P denote the circle with center at A which passes through P. For each positive integer n let α_n denote a set of n straight line intervals $A_{1n}B_{1n}, A_{2n}B_{2n}, \ldots, A_{nn}B_{nn}$ such that the points $A_{1n}, A_{2n}, \ldots, A_{nn}$ are equally spaced on J_{A_n} and $B_{1n}, B_{2n}, \ldots, B_{nn}$ are on J_{B_n} and, for each i ($1 \leqq i \leqq n$), A_{in} lies between B_{in} and A. Let K_n denote the continuum obtained by adding together J_{A_n}, J_{B_n} and all the intervals of the set α_n. Let K denote the point set $A + K_1 + K_2 + K_3 + \ldots$. Let G denote the collection of all continua g such that either g is A or, for some n, g is K_n or, for some point P of $T - K \cdot T$, g is J_P. Let M denote the sum of all the continua of the collection G. The point set M is a continuous curve and G is an arc with respect to its elements and its elements are arcatomic subsets of M. But M is a triod.

THEOREM 9. *Every compact irreducible continuum between two points has dendratomic subsets and they are also its arcatomic subsets.*

Theorem 9 is a consequence of theorem 6 and the fact that no irreducible continuum between two points is a triod.

[1] See page 357 of my book "Foundations of Point Set Theory," *Am. Math. Soc. Colloquium Pub.*, Vol. XIII, New York (1932).

[2] See my paper, "Concerning Triodic Continua in the Plane," *Fundamentae Mathematicae*, **13**, 262 (1929) and R. H. Sorgenfrey's as yet unpublished doctoral dissertation titled "Concerning Triodic Continua."

[3] An arcatomic subset of M will be defined if, in the above definition of a dendratomic subset of M, "dendron" is replaced by "arc" and "dendratomic" is replaced by "arcatomic."

CONCERNING WEBS IN THE PLANE

BY R. L. MOORE

DEPARTMENT OF PURE MATHEMATICS, UNIVERSITY OF TEXAS

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In my paper[1] "Concerning Continua Which Have Dendratomic Subsets," I defined webs and webless continua. As may be seen from the following example, it is not true that every continuum which is not a web is webless.

Example. In Euclidean space of three dimensions let E denote a plane, let J denote a circle lying in E, let I denote the bounded component of $E - J$ and let K denote $I + J$. Let T denote a connected point set topologically equivalent to a ray and such that T and E are mutually exclusive but $\overline{T} = T + K$. The point set $\overline{T}$ is an irreducible continuum from K to some point not lying in E. This continuum is not a web. But it contains the web K.

However, the following theorem holds true.

THEOREM 1. *In the plane, if a compact continuum contains a web then it is a web.*

Proof. In a Euclidean space Σ of two dimensions suppose M is a compact continuum containing a web L. There exist two upper semicontinuous collections H_1 and H_2 of mutually exclusive continua such that each of these collections fills up L and such that each of them is an arc with respect to its elements and every continuum of H_1 intersects every continuum of H_2.

Suppose t is a component of the common part of a continuum x_1 of the collection H_1 and a continuum x_2 of the collection H_2. Let y_1 denote a continuum of H_1 distinct from x_1 and let y_2 denote a continuum of H_2 distinct from x_2. Since y_1 intersects y_2, $y_1 + y_2$ is a continuum. Clearly this continuum is a subset of $L - t$. Let N denote $L - (x_1 \cdot x_2)$. Suppose P is a point of N. There exists a continuum p containing P, lying in N and belonging either to H_1 or to H_2. If p belongs to H_1 it intersects y_2 and if it belongs to H_2 it intersects y_1. Hence in either case it intersects $y_1 + y_2$

and therefore $p + y_1 + y_2$ is a connected subset of N containing P. It follows that N is connected. But every component of a proper closed subset of a compact continuum contains a limit point of that continuum minus that subset. Hence every component of $x_1 \cdot x_2$ contains a limit point of the connected point set N. Therefore $L - t$, the sum of N and all such components other than t (if there are any) is connected.

For each component t of the common part of a continuum of H_1 and a continuum of H_2, let h_t denote t plus the set of all points X of M, if there are any, such that t separates X from $L - t$. Let H denote the set of all such continua h_t. For each i ($i = 1, 2$) let H_i' denote the set of all sets h' such that, for some element h of H_i, h' is the set of all h_t's for all t's which are subsets of h. Let S' denote the collection of all continua P' such that P' is either a continuum of the collection H or a point which neither belongs to any continuum of H nor is separated by any continuum of H from any other continuum of H. If the elements of S' are regarded as "points" and the subcollection Z of S' is regarded as a "region" if and only if Z^* is a domain with respect to $(S')^*$ then Axioms 0, 1′, C, 2, 3, 4 and 5 of my book[2] *Foundations of Point Set Theory* all hold true for the space Σ_1 so obtained and, *in this space*, H is a continuous curve with no cut point and there exist a simple domain I, a simple closed curve J, four points A, B, C and F and two collections G_1 and G_2 satisfying all the requirements of the hypothesis of theorem 9 of page 380 and such that, for each i ($i = 1, 2$) every continuum of the collection G_i is a subcontinuum of some continuum of the collection H'_i. By a modification[3] of the argument given on pages 380 and 381 to prove theorem 9 it may be shown that, in the space Σ_1, there exists an upper semicontinuous collection G of mutually exclusive continua such that (*a*) if the elements of G are regarded as points and the subcollection Z of G is regarded as a region if and only if Z^* is a domain with respect to G^* then the space Σ_2 so obtained satisfies Axioms 0, 1′, C, 2, 3, 4 and 5, (*b*) in Σ_1 every non-degenerate continuum of the collection G is a subset of H plus the set of all elements of S' which are points of M which in Σ belong to no continuum of the collection H, (*c*) if, in Σ_1, g is a continuum of the collection G and P is an element of S' which either belongs to H or is, in Σ, a point of M which belongs to no continuum of the collection H then, in Σ_1, g does not separate P from any other continuum of the collection G, (*d*) if P is an element of S' which either belongs to H or is a point of M which, in Σ, belongs to no continuum of H then P either belongs to G or is, in the space Σ_1, an element of some continuum of the collection G, and (*e*) in the space Σ_2 there exists a connected domain D with a connected boundary β such that every degenerate element of G that belongs to D is, in Σ, a point of M or a continuum of the collection H. In the space Σ_2 let W denote the collection whose degenerate elements are the points of D and whose only non-degenerate element is the continuum γ obtained by adding to β

all points of Σ_2 which do not belong to D and which in Σ_1 are points of H or points which in Σ belong to M but to no continuum of the collection H. In Σ_2, W is an upper semicontinuous collection of mutually exclusive continua. There exists a reversibly continuous transformation throwing the continua of the collection W into the points of a sphere in a Euclidean space E of three dimensions. But clearly a sphere in E is a web. It follows that W is a web with respect to its elements. Hence, in the space Σ_2, there exist two upper semicontinuous collections of mutually exclusive continua Z_1 and Z_2 such that (1) each of these collections fills up $D + \gamma$, (2) each of them is an arc with respect to its elements, (3) each element of Z_1 intersects each element of Z_2 and (4) one element of Z_1 contains γ. For each continuum z of the collection Z_i $(i = 1, 2)$ let l_z denote the continuum in the space Σ_1 obtained by adding together all the continua in that space which are points of z in the space Σ_2 and let t_z denote the continuum in the space Σ obtained by adding together all the continua in the space Σ which are points of l_z in the space Σ_1. Let T_i denote the collection of all such t_z's for all elements z of Z_i. *In the space* Σ, for each i, T_i is an upper semicontinuous collection of mutually exclusive continua filling up M, T_i is an arc with respect to its elements and every continuum of the collection T_1 intersects every one of the collection T_2. Hence M is a web.

THEOREM 2. *If in the plane, M is a compact continuum, G is an upper semicontinuous collection of mutually exclusive continua filling up M and H is another such collection and, for every two continua g and h belonging to G and H, respectively, $g \cdot h$ exists and is totally disconnected then each of the collections G and H is a continuous curve with respect to its elements.*

Proof. Since every continuum of the collection G intersects every one of the collection H, therefore there exists a positive number e such that every continuum belonging either to G or to H is of diameter more than e.

Suppose M is not a continuous curve. With the help of Theorem 8 of chapter II it may be shown that there exist a sequence $M_1, M_2, M_3, \ldots$ of mutually exclusive subcontinua of M and a simple closed curve J containing four points A, B, C and D such that $A + C$ separates B from D on J and such that if I denotes the interior of J and, for every two points X and Y of this set of four, XY denotes the arc of J from X to Y which contains neither of the remaining ones then (1) the diameter of J is less than e, (2) for each n, M_n is a component of $M \cdot \bar{I}$ and M_{n+1} separates AD from M_n in $\bar{I}$. Let C_1D_1 denote an arc lying wholly in I except for its end-points C_1 and D_1 which lie between B and C and between A and D, respectively, on the arcs BC and AD. Let H' denote the set of all point sets x such that x is a component of the common part of $\bar{I}$ and some continuum of the collection H. For each n, M_n contains a point P_n of C_1D_1 and there exists a continuum h_n' belonging to H' and containing P_n and therefore lying in M_n. For each n, h_n' intersects either AB or CD. It follows that there exist two points

A_1 and B_1 and an infinite ascending sequence of positive integers n_1, n_2, n_3, . . . such that (1) either A_1 is D and B_1 is C or A_1 is A and B_1 is B and (2) for every j, h'_{n_j} intersects A_1B_1. Let I_1 denote the component of $I - I \cdot (C_1D_1)$ whose boundary contains the arc A_1B_1. Let C_2D_2 denote an arc lying wholly in I_1 except for its end-points C_2 and D_2 which lie between B_1 and C_1 and between A_1 and D_1, respectively, on the arcs BC and AD. Let G' denote the set of all point sets x such that x is a component of the common part of $\bar{I}_1$ and some continuum of the collection G. There exist points A_2 and B_2 and an infinite ascending subsequence m_1, m_2, m_3, ... of the sequence n_1, n_2, n_3, . . . such that (1) either A_2 is D_1 and B_2 is C_1 or A_2 is A_1 and B_2 is B_1, (2) for each j there is a continuum g_{m_j} belonging to G' and intersecting both A_2B_2 and C_2D_2 and lying in M_{m_j}. Let I_2 denote the component of $I_1 - I_1 \cdot (C_2D_2)$ whose boundary contains A_2B_2 and C_2D_2. For each j there exist two continua g_j and h_j such that (1) each of them intersects both A_2B_2 and C_2D_2, (2) g_j is a component of $\bar{I}_2 \cdot g'_{m_j}$ and h_j is a component of $\bar{I}_2 \cdot h'_{m_j}$. The sequence $g_1, g_2, g_3, \ldots$ converges to a continuum L and $h_1, h_2, h_3, \ldots$ converges to the same continuum. Hence there exist two continua g and h, belonging to G and H, respectively, such that L is a subset of each of them. But, since it intersects both A_2B_2 and C_2D_2, L is non-degenerate. This involves a contradiction. Hence M is a continuous curve. Therefore G is a continuous curve with respect to its elements and so is H.

If, in the statement of theorem 2, the words "in the plane" are replaced by "in Euclidean space of three dimensions," the resulting proposition is false.

Example. In Euclidean three dimensional space let AB denote a straight-line interval of length 1, let K denote a cube having AB as one of its edges and let F and L denote the two faces of K that contain AB. Let T denote the point set consisting of K plus its interior. For each n, let F_n denote the common part of T and a plane parallel to and at a distance equal to $1/n$ from, the plane in which F lies. Let M denote the continuum $F + L + F_1 + F_2 + F_3 + \ldots$ Let G denote the collection of all straight-line intervals g of length 1 lying in M such that g either coincides with AB or lies on a straight line parallel to the one containing AB. Let H denote the collection of all continua h such that h is the common part of M and some plane perpendicular to AB. The collections G and H are upper semicontinuous, each of them fills up M and if g and h are continua belonging to G and H, respectiely, $g \cdot h$ is a single point. The collection H is an arc[5] with respect to its elements. But G is not a continuous curve with respect to its elements and M is not a continuous curve.

[1] These PROCEEDINGS, **29**, 384–389 (1943).

[2] *Amer. Math. Soc. Colloquium Pub.*, New York (1932). References in this article to pages and chapters are to pages and chapters of this book. In the present connection see page 382.

[3] This argument is to be modified as follows: After the second sentence interpolate "Suppose either X denotes A and Y denotes C or X denotes B and Y denotes F. If X belongs to T let r_X denote XY plus the continuum of the collection G that contains X. If the interval t of the collection T has one end-point at X, let r_T denote $t + x_t + y_t$ where x_t is the continuum of the collection T that contains the other end-point of t and y_t is either XY or XY plus the continuum of G_1 that intersects XY according as XY and G^*_1 are or are not mutually exclusive." In the third sentence, after "T", interpolate "distinct from A and from B." Instead of the sentence beginning in the 18th line of page 381, write "In the space Σ_1, for each continuum q of the collection Q, let g_q denote the boundary of q, plus the set of all points of q, if there are any, which either (a) belong to H or (b) are points of M which in Σ belong to no continuum of the collection H. Let G denote the collection of all such g_q's."

[4] See theorem 16 of chapter V.

[5] Let M denote a continuum formed by a sequence of right pyramids all with the same square base α, their vertices converging to that of the outermost one. Let t_1 and t_2 denote two adjacent sides of α and let H_i denote the set of all intersections of M with planes perpendicular to t_i. Both H_1 and H_2 are arcs with respect to their elements but M is not a continuous curve.

EFFICIENT COMPUTATION OF THE LATENT VECTORS OF A MATRIX

BY PAUL A. SAMUELSON

DEPARTMENT OF ECONOMICS AND SOCIAL SCIENCE, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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In statistics, in quantum mechanics and in the study of dynamical oscillations it is often necessary to compute the latent roots and vectors of a matrix. A variety of methods are available for this purpose, involving direct algebraic computation, iteration and the application of perturbation-variation methods. It is the author's tentative conclusion from experience that the last two methods are excellent if only a few latent roots and vectors are desired, say those corresponding to the lowest or highest few roots, or if there exists some *a priori* familiarity with the data which permits very good initial guesses to be made. But in the general case of high order matrices both the iteration methods and the perturbation-variation methods become tedious, and so recourse must be had to direct algebraic computations.

A variety of methods are available under this heading, and the computer will choose between them not on the basis of their adequacy on constructed text-book examples, but in terms of a careful count of the number of calculations involved in each. Every method must involve the solution of a polynomial of the nth degree; but in addition, all of the methods known

to the present writer seem to involve multiplications which increase with the fourth power of n, where the matrix involved is of order n by n. It is the purpose of this note to present, perhaps for the first time, a method which gives latent vectors as well as latent roots after multiplications which increase with the third power of n.

1. Description of Procedure. Let a be the n by n matrix in question, and let h be an arbitrary column vector. Form the matrix products $(h_0, h_1, h_2, \ldots, h_n)$ by means of the operations $[Ih, ah, a(ah), \ldots, a(a^{n-1}h)]$. Then in consequence of the Cayley-Hamilton theorem that a matrix satisfies its own characteristic equation, we derive the coefficients of the characteristic equation $(1, p_1, p_2, \ldots, p_n)$ by solution of the following n linear equations

$$[h_0, h_1, \ldots, h_{n-1}]\begin{bmatrix} p_{n-1} \\ p_{n-2} \\ \cdot \\ \cdot \\ \cdot \\ p_1 \end{bmatrix} = -\, h_n.$$

Then let the characteristic equation, $f_n(X) = \sum_0^n p_j X^{n-j} = 0$, be solved by any method for the latent roots $(X_1, X_2, \ldots, X_n)$, assumed for simplicity to be distinct. We now form new polynomials by the relations

$$\begin{aligned} f_{n-1}(X) &= X^{n-1} + p_1 X^{n-2} + \ldots + p_{n-1} \\ f_{n-2}(X) &= X^{n-2} + p_1 X^{n-3} + \ldots + p_{n-2} \\ &\cdot \\ &\cdot \\ &\cdot \\ f_1(X) &= X + p_1 \\ f_0(X) &= 1 \end{aligned}$$

where each is formed from the previous by dropping off the last term and lowering the degree of the remaining terms. Expressions of the form $f_i(X_j)$ are easily computed as partial remainders in the familiar process of synthetic division.

Then the n latent vectors of a, $(V_1, V_2, \ldots, V_n)$, can be shown to be given by the product of the following two square matrices

$$V = [h_0, h_1, h_2, \ldots, h_{n-1}]\,[f_{n-i}(X_j)]. \qquad (1)$$

Should the original column vector, h, have been a linear combination of less than n latent vectors, the above process will fail; however, the probability of this occurring is very small, and such occurrences can easily be detected and allowed for. There is no reason why complex latent roots

and vectors cannot be handled in the above process. In the important special case where a is symmetrical, only real quantities will be involved, and a check upon the numerical computations is provided by the conjugate property $V_j'V_i = 0$, for $i \neq j$. When repeated roots are encountered, the modifications are relatively minor.

The labor involved in numerical processes of the above type is best reckoned in terms of the required number of multiplications. The method described here involves multiplications of the order $8n^3/3$, or about the equivalent of three square matrix multiplications. In addition, one nth degree polynomial must be solved. It will be noted that each latent vector can be determined independently of all the rest, once its corresponding latent root has been determined. Approximate values of a latent vector can be computed from the insertion of approximate roots in the process of synthetic division indicated above.

2. *Proof.* Consider the system of differential equations written in matrix form

$$DY(t) = aY(t).$$

If the latent roots are all distinct, it is known that the solution of these equations for initial values $Y(0) = h$ is unique and given by

$$\begin{aligned} Y_1(t) &= c_{11} \exp X_1 t + c_{12} \exp X_2 t + \ldots + c_{1n} \exp X_n t \\ Y_2(t) &= c_{21} \exp X_1 t + c_{22} \exp X_2 t + \ldots + c_{2n} \exp X_n t \\ &\cdot \\ &\cdot \\ &\cdot \\ Y_n(t) &= c_{n1} \exp X_1 t + c_{n2} \exp X_2 t + \ldots + c_{nn} \exp X_n t. \end{aligned}$$

The n^2 c coefficients are not all arbitrary, only n of them being dependent upon the initial conditions. Each column of the c's is equivalent to the appropriate latent vector, the initial conditions simply determining the factors of proportionality of the latent vectors.

Our task then is to compute the solution of such a set of differential equations; from this solution we can easily identify the appropriate latent vectors. Thus, we reverse the usual procedure in which the latent vectors are first algebraically computed as an aid in giving the solution of the differential equation system. The novelty of the present method consists in the recognition of this fact plus the specification of a speedy method of arriving at a particular solution of the differential equation system. It is fashionable to handle this last problem by means of the Heaviside-Cauchy operational calculus; a careful consideration of these techniques from a computational point of view will show their efficiency to be greatly overrated, involving in this case multiplications of the order n^4.

It is a commonplace that an nth order differential equation in one

variable can be transformed into n first order equations. It is no less true that a system of the latter form can be converted into single equations of the nth order in each variable; for constant coefficient systems such as the one under consideration, the coefficients of the differential equation are in each case simply the coefficients of the characteristic equation; i.e.,

$$f_n(D)\,Y_j(t) = 0. \qquad (j = 1, \ldots, n).$$

If we can identify the appropriate initial conditions for each of the last equations, and then give the solution of each, we should end up with the required c coefficients indicated above. As for the appropriate initial conditions, if $Y(0) = h$, then by repeated use of the original differential equations, it becomes evident that

$$D^i Y(0) = a^i h = h_i.$$

It is a classical fact that the solution of an nth order differential equation for given initial conditions is given by the solution of a set of linear equations whose matrix is of the Vandermonde-Cauchy form

$$\begin{bmatrix} 1 & 1 & \cdots & 1 \\ X_1 & X_2 & \cdots & X_n \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ X_1^{n-1} & X_2^{n-1} & \cdots & X_n^{n-1} \end{bmatrix},$$

the transposed inverse of which is given by

$$\begin{bmatrix} \dfrac{P_{n-1}(X_1)}{P_n'(X_1)} & \dfrac{P_{n-1}(X_2)}{P_n'(X_2)} & \cdots & \dfrac{P_{n-1}(X_n)}{P_n'(X_n)} \\ \dfrac{P_{n-2}(X_1)}{P_n'(X_1)} & \dfrac{P_{n-2}(X_2)}{P_n'(X_2)} & \cdots & \dfrac{P_{n-2}(X_n)}{P_n'(X_n)} \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \cdot & \cdot & & \cdot \\ \dfrac{1}{P_n'(X_1)} & \dfrac{1}{P_n'(X_2)} & \cdots & \dfrac{1}{P_n'(X_n)} \end{bmatrix}$$

Consequently the n by n c matrix, which is also the matrix made up of columns of latent vectors is given by equation (1) above, where the factors of proportionality $1/P_n'(X_i)$ have been omitted.

When repeated roots are encountered, a generalized Vandermonde determinant is involved whose inversion is easily effected if one simply pursues the close analogy between Vandermonde determinants and expansions in partial fractions.

If the same differential equations are to be solved for many different initial conditions, the above process may be repeated anew. Or a slight economy of effort may be achieved if the inverse of V is worked out once and for all so that the weightings of the different exponential terms can be easily determined by $V^{-1}h$. If the a matrix is symmetrical, the latent vector matrix will be orthogonal so that simple transposition will provide the inverse matrix, except for factors of proportionality.

A SIMPLE METHOD OF INTERPOLATION

By Paul A. Samuelson

Department of Economics and Social Science, Massachusetts Institute of Technology

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I.—In many branches of statistics it is necessary to determine the coefficients of an nth degree polynomial, $f(x)$, from $n + 1$ observations, $(x_0, f_0;\ x_1, f_1;\ \ldots;\ x_n, f_n)$, and to determine readings from this polynomial. For this latter purpose recourse may be had to divided differences, Lagrange's interpolation formula,[1] Aitken's method of interpolation,[2] etc. However, where a number of readings are to be taken, or where the coefficients are of interest for their own sake, it is necessary to solve a system of linear equations

$$\begin{aligned} a_0 + x_0 a_1 + x_0^2 a_2 + \ldots + x_0^n a_n &= f_0 \\ \ldots\ldots\ldots\ldots\ldots\ldots\ldots & \quad \text{or } Va = f \\ a_n + x_n a_1 + x_n^2 a_2 + \ldots + x_n^n a_n &= f_n \end{aligned}$$

whose matrix is of the familiar Vandermonde form (x_i^j).

Now in the solution of nth order differential equations with constant coefficients and one-point boundary conditions, such as occur in electrical engineering and other fields of applied mathematics, the solutions can be written in the form of linear combinations of particular solutions, the coefficients being determined by the solution of a transposed Vandermonde set of linear equations.

By means of the Heaviside-Cauchy operational calculus (Laplace transform, etc.), the applied mathematician is able to avoid explicit inversion of such a system of equations. This suggests the possibility of lessening the calculations involved in interpolation by methods analogous to those used in solving differential equations; and upon examination it turns out that the resulting method seems admirably suited to numerical computation, with or without a modern calculating machine.

II.—For those interested simply in the procedure, we first describe the workings of the method, reserving to the last section the question of proof. The work is divided into three stages.

First, an auxiliary polynomial of the $(n + 1)$th degree is determined so that its leading coefficient is unity, and so that it vanishes at $(x_0, x_1, \ldots, x_n)$. Instead of working out its coefficients from the relations which must hold between the roots and coefficients of a polynomial, it is far simpler in practice to compute successively the partial products $\prod_0^r (x - x_i)$. The tabular arrangement in the numerical example suggests how this may be done expeditiously. We may write this polynomial as

$$P_{n+1}(x) = \prod_0^n (x - x_i) = x^{n+1} + b_1 x^n + \ldots + b_n x + b_{n+1}.$$

We associate with this, polynomials defined as follows

$$\begin{aligned} P_n(x) &= x_n + b_1 x^{n-1} + \ldots + b_{n-1} x + b_n \\ &\cdots\cdots\cdots\cdots\cdots\cdots \\ P_1(x) &= x + b_1 \\ P_0(x) &= 1. \end{aligned}$$

Each is derived from the preceding by dropping the last coefficient and lowering the power in each of the remaining terms.

In terms of the above, the unknown coefficients of $f(x)$ are given by the expressions

$$a_j = \frac{P_{n-j}(x_0)}{P_{n+1}'(x_0)} + \frac{P_{n-j}(x_1)}{P_{n+1}'(x)} + \ldots + \frac{P_{n-j}(x_n)}{P_{n+1}'(x_n)}. \qquad (1)$$
$$(j = 0, 1, 2, \ldots n)$$

The second stage of the numerical work consists of an evaluation of terms like $P_j(x_k)$ and $P_{n+1}'(x_k)$. This can be easily done by the familiar algebraic device of synthetic devision, which lends itself very well to machine calculation. From the definition of the P's, it follows that the partial remainders at each stage of synthetic division of P_{n+1} by x_k are, respectively, $P_0(x_k)$, $P_1(x_k)$, $\ldots$, $P_n(x_k)$, $P_{n+1}(x_k)$. Thus, all are calculated at once in the same operation. Moreover, by definition the last of these must in every case vanish, providing a check upon the accuracy of the previous work.

After the above terms have been calculated by synthetic division, a second synthetic division performed upon them gives in the penultimate position the required derivative $P_{n+1}'(x_k)$, in a manner familiar to all users of Horner's method.

The third stage consists simply of the summation indicated in equation (1). Some economies of keyboard or slide-rule settings can be realized if the indicated divisions are done all at once wherever possible.

The amount of effort required in the proposed method may be compared with other methods. To invert the linear equations directly would require multiplications of the order $2n^3/3$ if efficient Gauss-Doolittle methods are used. If advantage is taken of the Vandermonde form of the matrix as in the Aitken method, the calculations of a single reading seem to require multiplications and divisions of the order $3n^2/2$, and, of course, the coefficients of the polynomial are not derived.

The present method entails multiplications of the order of $3n^2$ plus n multiplications for each reading, where the method of synthetic division is used to derive the readings. If two readings are desired, the work is therefore about equal to the Aitken method, and for more than two readings it is very much less.

After using the method for awhile, one will learn how to effect certain economies of labor which need not be indicated here. Thus, if the abscissa points are all positive, as often occurs in practice, the occurrence of negative numbers in the calculations may be lessened by a reversal of signs, followed by a determination of $f(-x)$.

III.—The procedure may be illustrated by a simple numerical example. Suppose we are to fit a quadratic polynomial to the following three points $(-1, 4;\ 0, 1;\ 2, 7)$. Clearly the correct answer is given by $f(x) = 2x^2 - x + 1$. To achieve this by the present method, the coefficients of the partial products are computed in the following tabular arrangement.

$$\begin{array}{cccc:c} 1 & 1 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & -2 \\ 1 & -1 & -2 & 0 & \end{array}$$

Each element in a given row is computed from the elements of the preceding row by adding to the element directly above it the product of the element just northwest of it times the number in the preceding row just to the right of the colon. The last row of all gives the coefficients of the auxiliary cubic polynomial.

The second stage of the numerical work consists of dividing this polynomial in turn by $(x + 1)$, x, and $(x - 2)$, so as to calculate the partial remainders. Arranging the work as in synthetic division

$$\begin{array}{cccc|c} 1 & -1 & -2 & 0 & -1 \\ 1 & -2 & 0 & 0 & -1 \\ 1 & -3 & +3 & & \end{array}$$

The second line gives the successive values of $P_i(-1)$. The last recorded item in the third row gives $P_3'(-1)$. If a modern calculating machine is used, only the final element in the third row need be copied.

This process is repeated for each of the roots, without however, having

to recopy the starred first rows. Thus,

$$\begin{array}{rrrrr} *1 & -1 & -2 & 0 & \underline{|0} \\ 1 & -1 & -2 & 0 & \underline{|0} \\ 1 & -1 & -2 & & \\ *1 & -1 & -2 & 0 & \underline{|2} \\ 1 & +1 & 0 & 0 & \underline{|2} \\ 1 & 3 & 6 & & \end{array}$$

The coefficients in each of the second rows is divided by the last element in the corresponding third row, and when arranged in order give us the inverse of V'.

$$(V')^{-1} = \begin{bmatrix} 1/3 & -2/3 & 0 \\ -1/2 & 1/2 & 1 \\ 1/6 & 1/6 & 0 \end{bmatrix}$$

Premultiplying this by the row matrix $(f_0, f_1, \ldots, f_n)$, or in this case by (4, 7, 1), the operations described in (1) are carried out, yielding the requisite solution (2, −1, 1).

Two partial checks on the accuracy of the work may be mentioned. The divisors, being derivatives at simple roots of the auxiliary polynomial should oscillate in sign, with the last one being positive. Also, the sum of the elements in the last column of the inverse matrix should add up to unity, while the other columns should add up to zero. This provides a check upon all earlier operations. Of course, a final decisive check is provided by evaluating the resulting polynomial to verify that it does go through the prescribed points. This is best done by synthetic division.

IV.—To justify the method it is only necessary to show that the elements of the inverse of the transposed Vandermonde matrix are, in fact, equal to $P_{n-j}(x_i)/P_{n+1}'(x_i)$. This could be done directly by means of contour integration or by the elementary properties of symmetric functions. An indirect proof which lends itself readily to the generalization given below involves the fact that the expression above can easily be shown to give the coefficient of certain terms in the operational solution of differential equation systems. At the same time the classical non-operational solution yields the corresponding coefficients in the form of the inverse of the Vandermonde matrix. Since the classical and operational solutions can easily be shown to be identical, it follows that our theorem must be true.

This suggests a slightly more general method of interpolation in case we are to find a polynomial from a knowledge of its values, and the value of its derivatives up to some varying order, at a number of points. As in the above case, synthetic division, now carried to more rows, is utilized,

and the work of fitting a polynomial of the same degree is only slightly increased.

The matrix of the equations which must be solved now takes the general partitioned form

$$V' = \left[\begin{array}{cccc|c|c|ccc} 1 & 0 & & & 1 & & 1 & & \\ x_0 & 1 & & & x_1 & & x_r & & \\ x_0^2 & 2x_0 & & & x_1^2 & & & & \\ \cdots & \cdots & \cdots & \cdots & \cdots & & & & \\ x_0^j & (x_0^j)' & \cdots & (x_0^j)^{m_0-1} & x_1^j & \cdots & (x_1^j)^{m_1-1} & x_r^j & \cdots & (x_r^j)^{m_r-1} \\ \cdots & \cdots & \cdots & \cdots & \cdots & & & & \\ x_0^n & nx_0^{n-1} & \cdots & \dfrac{n!x_0^{n-m_0+1}}{(n-m_0+1)!} & x_1^n & & & & \end{array}\right]$$

where $(x_s^j)^v = \dfrac{d^v}{dx^v}[x^j]_{x=x_s}$.

Then by precisely the same kind of proof as that sketched above, it can be shown that the inverse of this generalized Vandermonde matrix is given by

$$(V')^{-1} = \left[\frac{1}{(m_i-k)!k!}\frac{d^{m_i-k}}{dx^{m_i-k}}\left\{\frac{P_{n-j}(x)}{P_{n+1}(x)/(x-x_i)^{m_i}}\right\}_{x=x_i}\right]$$

where

where $P_{n+1}(x) = \prod_0^r (x-x_j)^{m_j} = x^{n+1} + b_1x^n + \ldots + b_{n+1}$

$P_n(x) = x^n + b_1x^{n-1} + \ldots + b_n$

etc.

[1] Whittaker, E. T., and Robinson, G., *The Calculus of Observations*, Chap. II, Blackie and Son, Glasgow, 1932.

[2] Aitken, A. C., "On Interpolation by Iteration of Proportional Parts without the Use of Differences," *Proc. Edinburgh Mathematical Society Series*, **2**, No. 3, 56–76 1932.

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